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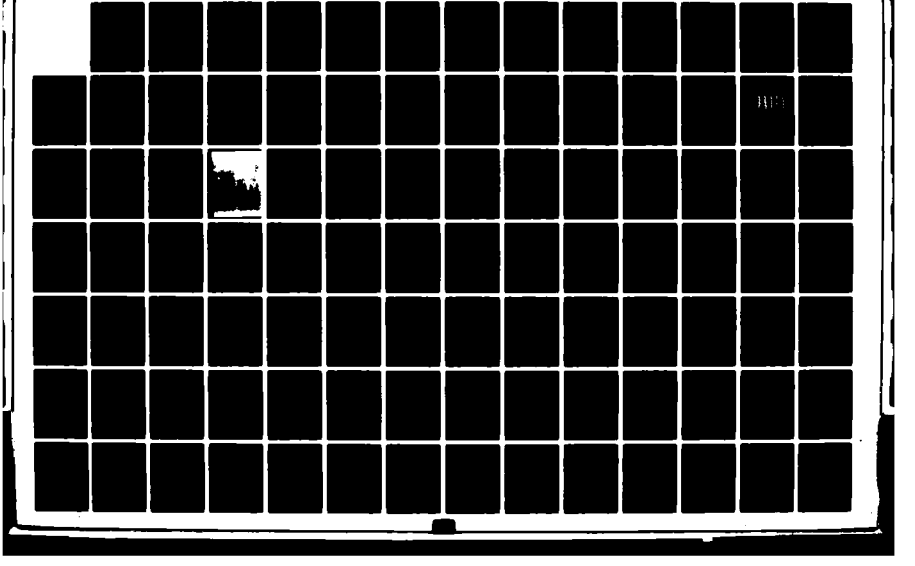

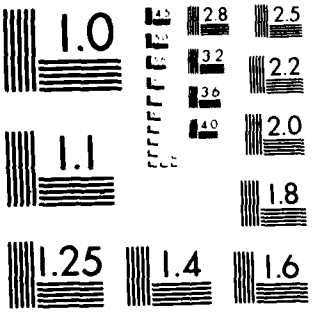

OFFICE OF NAVAL RESEARCH - BIOPHYSICS PROGRAM - RESEARCH PROPOSAL

1. TITLE OF RESEARCH PROPOSAL:

"Molecular Mechanisms Involved in Tissue Swelling Due to Injury and Due to Exposure to Low Temperature and Massive Water and Electrolyte Loss in Diarrheal Disorders"

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MOLECULAR MECHANISMS INVOLVED IN TISSUE SWELLING DUE TO INJURY AND DUE TO EXPOSURE TO LOW TEMPERATURE AND MASSIVE WATER AND ELECTROLYTE LOSS IN DIARRHEAL DISORDERS

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OBJECTIVES

The goal of this study is to understand the molecular mechanisms underlying swelling of brain and other tissues as a result of injuries and due to exposure to low temperature and the underlying massive loss of water and electrolytes in various diarrheal diseases.

ABSTRACT

A serious cause of death in brain injury is due to extensive swelling. This swelling of brain as well as a variety of other mouse tissues can be traced to failure to maintain their normal ATP level. More importantly, it was shown that swelling will not occur even after ATP depletion unless the tissues are in an environment containing Na^+ (and Cl^-) as it is in normal plasma. A quantitative relation has been discovered between the concentration of Na^+ in the surrounding medium and the extent of swelling in brain (and other tissues) whose ATP levels have been reduced by poisons or exposure to low temperature. These findings are in full harmony with a theory of the living cells called the association-induction hypothesis, according to which the Na^+, Cl^-, and ATP dependent swelling is due to dissociative effect of salt linkages normally maintaining the cell volume. Other studies confirm the theoretical concepts that certain cells such as the intestinal epithelium conserve body water not due to a postulated lipid bilayers but are due to water existing in the state of polarized multilayers. Such polarized water has reduced solubility for the major osmotically active component of the plasma, Na^+ (and Cl^-), toxins induce diarrhea as a result of depolarization of water of the intestinal epithelial cells and an increase in the permeability of Na^+ (and Cl^-) through the barrier of water follows in consequence.

PLANS FOR THE FUTURE

The ONR- (and also NIH) sponsored programs of this laboratory have made possible a major change of the basic concept of the living cell: the three major components of all living cells, water, proteins, and K^+ are in close cooperative association rather than merely a solvent and solutes as it is the case in a Ringer solution. The new concepts fully described in another monograph expected to be in print in the next year, entitled, "In Search of the Physical Basis of Life," will be fully exploited both theo-

retically and experimentally to help understand and eventually to evolve methods to alleviate or cure ailments afflicting combat naval personnels. More specifically and as an illustration, we plan to pursue the study of brain swelling, with the aim of finding conditions that could alleviate harmful effects of brain (and other tissue) swelling and massive water and electrolyte loss in infectious diseases.

CURRENT REPORTS AND PUBLICATIONS

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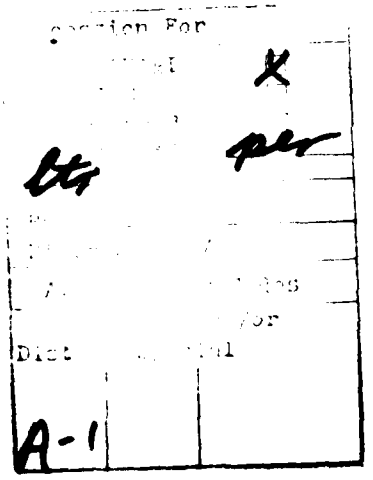
15. Ling, G.N. and Fisher, A.: Cooperative interaction among cell surface sites: Further evidence in support of the surface adsorption theory of cellular electrical potential. (in preparation).
16. Ling, G.N., and Walton, C.: Effect of external Mg^{+} concentration on the resting potential of frog muscle cell: A further test of the membrane theory vs. the surface adsorption theory. (in preparation).
17. Ling, G.N., and Tucker, M.: Only solid red blood cell ghosts transport K^{+} and Na^{+} against concentration gradients: Hollow intact ghosts with K^{+}-Na^{+} activated ATPase do not. (in preparation).
18. Ling, G.N., and Murphy, R.C.: Nuclear magnetic resonance relaxation of water protons under the influence of proteins and other linear polymers. (in preparation).
19. Ling, G.N.: Synchronous control of metabolic activity of living cells by K^{+} transiently and reversibly liberated from adsorption sites during physiological activities: A subsidiary hypothesis of the association-induction hypothesis. (in preparation)

Monograph

20. Ling, G.N.: In search of the physical basis of life. (in preparation)



Background Information

I. Long-range Ordering of Water

A. Theory and Significance

Living system differs from the non-living in its internal coherence. Physiologists have long emphasized two means whereby multicellular organisms provide such coordination: hormonal control and neurological control. However, there is another level of coordination that is more fundamental: control at the molecular level. It was in the 1962 version of the Association-Induction Hypothesis that a molecular and electronic mechanism was offered for the transmission of biological signals over long distances, referred to as the indirect F-effect. By means of this effect, interaction of a protein molecular at a cardinal site can be transmitted to a distant protein site. A 1965 article presenting the polarized multilayer theory of cell water, completes the theme: Since all living matter is roughly 80% water and in this theory, all the water is under the long-range control of certain proteins, a degree of molecular coherence is thus provided for the entire living cell to function as a coherent unit. With this in mind, it seems hardly an exaggeration to state that experimental verification of the polarized multilayer theory that the exposed NH and CO groups on extended protein chains can polarize deep layers of water is a matter of the most profound importance.

According to the polarized multilayer theory of cell water, a matrix of extended polypeptide chains with their NH and CO groups directly exposed to water, is capable of polarizing and orienting rows of water molecules in such a manner that each strand oriented in one direction is surrounded by neighboring strands oriented in the opposite direction. The result is a dynamic three dimensional lattice of cooperatively linked water molecules. This lattice is different from an ice lattice (i.e., Ice I) in that the structure is highly flexible rather than rigid and hence lacking the kind of long-range order of a true crystal lattice. Nevertheless such a long range order though blurred does exist if one averages the observation over long period of time; in this it differs from normal liquid water, which if observed over long periods of time, would show no structure.

Long-range ordering of water has been investigated in many ways and with many sophisticated methods. None of these offers a degree of clean-cut unequivocalness essential for establishing this fundamental theory. It is with this historical background in mind that the "q-value technique" we developed stands all by itself:

(1) It offers a means to tell precisely and unequivocally how many water molecules at least are influenced. (2) It recognizes the very same property of water for whose explanation in the living cells the long-range ordering of water was proposed. (3) It is extremely simple and straightforward.

The q-value technique can best be explained with an example: If a protein-water system in equilibrium with a pure water, the q-value (or equilibrium distribution coefficient between the protein water and water) of a probe molecules (e.g., Na^+, sucrose, or amino acid) is 0.4, then at least 0.6 or 60% of the water in the protein-water system must be under the influence of the proteins. This is so because a q-value of 0.4 is quantitatively equivalent to 60% of the water having no solubility at all for the probe molecules. In truth more than 60% of water molecules must be sufficiently altered because it is highly unlikely that the influenced water has zero solubility; therefore the protein-influenced water must be more than 60%. Hence the q-value yields a minimum number of water molecules influenced.

B. Experimental testing

Since in this polarized multilayer theory of cell water, long-range water polarization arises from the presence of extended polypeptide chains with the NH and CO groups directly exposed to water. Several corrolaries to this theory are as follows:

(1) Corollary 1: In whatever way if these NH, CO groups are otherwise engaged either in intrachain NH...OC-H bonding (e.g., in α-helical conformation) or in interchain NH...OC-H-bonding (e.g., in β-pleated sheet conformation), this portion of the protein chain would have no long-range water ordering effect.

(2) Corollary 2: Conversely, if a protein where backbone NH CO groups are so engaged and they have no long-range ordering effect on cell water, will acquire such a long-range ordering effect, if one can bring about a dissociation of these intra- as interchain H-bonding and exposing their NH CO groups directly to water.

(3) Corollary 3: The long-range ordering effect should not be limited to protein but is expected to apply to any matrix of linear polymer containing strong H-bonding groups at proper distance apart that do not form intra- or intercellular H-bonds.

(4) Corollary 4: In a system of linear chains, parallel orientation favors long-range ordering of water while random orientation and overlapping arrangements have the opposite effect.

As of this moment, from work still in progress, we can safely state that all these fundamental theoretical predictions have been verified.

1. Due to inter and intrachain H-bonding (largely α-helical) thirteen native globular proteins at concentrations from 15 to 25% in 1.5 M Na_2SO_4, shows consistency a q-value of 1.0 for the probe molecule Na^+ indicating no long-range ordering (Table 1).

2. Gelatin, a denatured product of collagen, on the other hand, due to its unusual amino acid composition containing large proportions of proline and hydroxyproline cannot form α-helices and, due to denaturation, forms only limited chain-to-chain H-bonds showing a q-value of 0.6 in 1.5 M Na_2SO_4. This low q-value establishes long range ordering of at least 40% of the water in the gelatin-water system (Figure 1).

3. Similarly several synthetic polymers including poly-vinylpyrrolidine with H-bonding sites at intervals similar to gelatin exhibit also long-range ordering ability (Figure 2).

4. Urea and guanidine HCl, known to denature proteins by disrupting the α-helical structures, are capable of converting non-water ordering native proteins into confirmations that do have long-range water-orienting property. (Figure 3)

5. Extreme pH are known to extend poly-lysines and poly-glutamic acids causing them to go from helical to non-helical state also converts them from being able to order water in long-range to another state that is unable to order water.

6. The protein denaturant sodium dodecyl sulfate and n-propanal, which denature proteins by disrupting only the tertiary structure but does not disrupt the α-helical structure or even enhance of α-helical contents, also does <u>not</u> convert native protein from non-water ordering to water ordering. (Figure 4)

II. Swelling and Shrinkage of Living Cells

A. History. The study of the living cells began with this subject; swelling in a hypotonic environment and shrinkage in a hypertonic environment. It was the demonstration that a solution of sugar and salt when enclosed in an artificial semipermeable membrane sac (especially the copper-ferrocyanide gel membrane of M. Traube) swells and shrinks in hypertonic and hypotonic solutions respectively in a manner qualitatively following what was later known as the Boyle-van't Hoff law that led Pfeffer to the founding of the membrane theory. The Boyle-van't Hoff law states that the product of the osmotic pressure P and the volume of the semipermeable membrane enclosed system, V, is a constant, i.e.,

$$PV = \text{Constant.} \qquad (1)$$

In other words, for the same system an increase of the osmotic pressure of the surrounding medium to twice its initial value should cause shrinkage to half of its initial size. Conversely a decrease of the osmotic pressure to ½ of initial value should cause a doubling of the value.

However, careful investigation soon led to the realization that strict obedience to the Boyle-van't Hoff law occurs only in plant cells with large central vacuoles. Indeed it was also found that part of these plant cells responsible for this behavior is the tonoplast (inner membrane surrounding the central vacuole) covered central vacuole. This tonoplast covered central vacuole was isolated by Hoeffler and Chambers and shown to follow in its osmotic behavior the Boyle-van't Hoff law. For cells without a central vacuole, e.g., muscle cell, the volume changes in hypotonic solutions fall far short of that predicted by the Boyle-van't Hoff law. This point was noted by no other than Pfeffer himself, who founded the membrane theory. However, it was Overton, another of the founding fathers of the widely taught membrane theory, who working with frog muscles came to the conclusion that this departure was too large to be ignored. To explain, he postulated that a part of the cell water does not participate in osmotic volume changes. This part of the water he referred to be "Schwellungswasser" - a term often used in the German lingo of colloidal chemistry of those days. "Schwellungswasser" meaning swelling water was translated, I believe, more correctly as "imbibition" water but more often as "bound water." Correctly translated or not, it was more important to appreciate Overton's suggestion as indicating his recognition of trouble with the membrane theory. (It was no more than a vague speculation that Schwellungswasser does not change with osmotic pressure alterations, see below). As such, Overton implied that only part of the water participates in the swelling and shrinkage, the rest does not. With this modification, the van't Hoff equation as applied to muscle swelling and shrinkage assumed the form

$$P\,(V-b) = \text{Constant}, \qquad (2)$$

where b is the volume of the Schwellungswasser. It may amount to 1/3 to 1/2 of the total cell water.

Overton's experimental observation was confirmed by A. V. Hill in a paper published in 1930. Historically it was this paper that led many biologists to the acceptance of the membrane theory and to the abandonment of the colloidal chemical approach to cell physiology. Hill's powerful influence reflected to a considerable extent his prestige as a scientist. Seen from the vantage point half a century later, his scientific argument was not all that convincing. This argument consists of two parts: first, his demonstration that all water in muscle cells can dissolve urea, thus proving that no substantial amount of water is "non-solvent" as "non-solvent" was stated by Gortner and other champions of the bound water concept to be its key feature. Second, Hill suggested that about 25% of the water in the muscles

had perished in its stay in the hyper- and hypotonic solutions. This is to all intents and purposes, where the matter remained to this date.

In fact most proponents of the membrane theory with the exception of those working specifically on cell water, have all but forgotten this issue which is nevertheless a matter of critical importance to the tenability of the membrane theory.

B. New findings

It has been a subject of continual interest to this laboratory to evolve better methods for the in vitro preservation of isolated adult, non-proliferative tissue cells. Thus we are able to maintain frog sartorius in good condition as judged by its electrical potentials, ionic contents and contractibility for at least 8 days at 25°C, a month or more at 0°C.

With this method, it is easy to conduct a swelling experiment much as Overton and Hill did a long time ago but in a much more favorable environment. It was shown volume changes reach their equilibrium at 0°C within 3 hours. Within limits (hypotonicity to 20% of isotonic), no significant cell death was observed; the Na^+ contents of the treated cells after return to equilibrium in normal Ringer solution are normal. If 25% of the cells were dead, the Na^+ content would have risen by at least 100% since dead cells contain somewhat higher Na^+ contents than that in the bathing Ringer solution while normal cells contain a small fraction of external Na^+. These simple experiments quite clearly show that Hill's interpretation of 25% cell death is certainly not the cause of departure of the swelling and shrinkage behavior of frog muscle cells.

Table 2 further shows what proportion of the muscle cell water (b-value) has to be "Schwellungswasser", or more correctly "non-water", in order for the data to fit the prediction of the membrane theory. Note that in some cases, the bulk of cell water has to be "non-water". Yet the distribution of urea (Hill, 1930) and of ethylene glycol (McLeod and Ponder, 1936) in muscle cells show that virtually all the water is capable of dissolving these water soluble materials. We have therefore reached a dead end for the membrane theory: the only two ways to explain the swelling and shrinkage of frog muscle cells: postulation of non-water (or Schwellungswasser) or dead cells are both untenable.

C. New Theory

According to the association-induction hypothesis, the retention of cell water is not due to the presence of a semipermeable cell membrane holding salt ions and water in the cell. Rather, it is primarily the result of the long-range ordering of (all or virtually all) the cell water by the extended polypeptide chains in a more or less regular matrix. The maintenance of the proteins essential for the long-range ordering of the cell water depends on the adsorption of ATP on certain cardinal sites. When ATP decomposes as in poisoned cells, the protein goes into a helical or other conformation with its H-bonding sites internally neutralized; as a result the ordering of water is abruptly removed and the water approaches in behaviors, those of normal liquid water.

(1) K^+ adsorption in muscle cells

This theory, therefore, is entirely compatible with the gathering evidence that the bulk of K^+ ion in the muscle cells, which make up virtually the

entirety of the intracellular ionic content, is osometrically inactive, being in the adsorbed state (Edelmann, 1977; Ling 1977). The same findings cannot be reconciled with the membrane theory according to which the bulk of intracellular ions must be as free as the NaCl in the Ringer solution in which the cells are immersed. In the membrane theory by far the largest source of osmotic strength arises from the intracellular ions, free amino acids, sugars, etc., not because they make up the bulk of the weight of non-water components of the cells but because their total osmolarity is large; and the single largest ionic constituent is K^+. Protein, which makes up the bulk of non-water component, is according to the membrane theory, of relatively trivial importance because its total molar concentration is very small due to their enormous molecular weights (1 or 2 mM).

(2) Indifference of osmotic swelling to the intactness of cell membrane

The swelling of muscle cells in a hypotonic solution is quantitatively the same whether the cells are intact or cut into small segments - 4 kinds of independent evidence showed that no membrane regeneration occurs (Ling and Walton, 1976). These findings are again incompatible with the membrane theory, but are in full harmony with the association-induction hypothesis.

New Experimental Findings

The association-induction hypothesis would be greatly strengthened if we can demonstrate in a macromolecule-H_2O system, which, without a semipermeable membrane, can nevertheless swell or shrink as a living cell does when bathed in a more dilute or more concentrated salt solution respectively. This would be most effective in confirming the association-induction hypothesis if the macromolecules satisfy the requirement of NP-NP-NP, or simpler, NO-NO-NO or PO-PO-PO system and that the macromolecules contain no fixed charges. This indeed is what we have succeeded in demonstrating. (For definition of NP-NP-NP system, etc., see Appendix)

Using conventional dialysis tubing to confine the non-charged polymer, we were able to show that as we varied the concentrations of Na_2SO_4 in the bathing solution, the water contents of the polymer-water system rise or fall in a manner also basically similar to that seen in the osmotic behavior of living cells (Fig. 5). This is shown as B of Figure 6: Where the water content expressed as gram of water per gram of dry polymer is plotted against the logarithm of $(1 - \frac{p}{P_o})$ where p is the vapor pressure of water and P_o the saturated vapor pressure at the same temperature. It should be noticed that to a first approximation

$$[1 - \frac{p}{P_o}] = \text{Constant} \cdot P \qquad (3)$$

when P is the osmotic pressure of the Na_2SO_4 solute bathing the polymer-filled dialysis bags. Thus Figure 6 is essentially a plot of water contents against the osmotic pressure.

An entirely similar, not only qualitatively but quantitatively similar, water content vs. $[1 - \frac{p}{P_o}]$ plot is obtained for frog muscles. The swelling or shrinkage of neither depends on the presence of semipermeable-membrane and are most readily explained as due to similar extended (protein) chains in the living muscle cells.

III. Swelling of Cold Injured Living Tissues

It is well known that injured tissues swell. The membrane theory proponents argue that this is due to damage to the Na pump, the failure of which leads to

accumulation of Na^+ in the cell and the enhanced osmotic pressure causes swelling. There are at least two reasons to believe that this theory is not tenable: (1) in an injured or dying muscle as a rule the gain of intracellular Na^+ is made up by an equivalent loss of intracellular K^+. There could not, therefore, be an excess of osmotic pressure in the cell to cause swelling. (2) injured or dead cells remain swollen even though the cell membrane has long become leaky.

On the other hand, injury-induced swelling has a rather interesting explanation that is derived from the core-concept of the association-induction hypothesis; as ATP declines there are broad electronic shifts within the protein molecules. One of these consequences is that β and γ carboxyl groups could alter their c-value to a high value. As a result, the high preference for K^+ and the closely similar fixed cationic group (ϵ - amino groups carried by lysine and guanidyl groups carried by arginine residues) over Na^+ is lost or diminished (see theoretical curve, shown in Figure 7).

One recalls (see ONR Progress Report of 1977) that a frog muscle soaked in a Ringer Solution whose NaCl is replaced by an equimolar concentration of KCl undergoes extensive swelling.

It was shown theoretically that this swelling reflects a decrease of the salt linkages ($f^- - f^+$) normally restraining the cytoplasm from expansion:

$$f - f^+ + K^+ + Cl^- \rightleftharpoons f^- K^+ + f^+ Cl^- \qquad (4)$$

when f^- represents fixed anionic sites (i.e., β- and γ-carboxyl groups) and f^+ represents fixed cationic sites (i.e., ϵ-amino group and guanidyl groups). It is to be noted that to be an effective swelling salt, not only has the cationic component (K^+) to be preferred by f^- but the anionic component (Cl^-) must be preferred by f^+. It is for this reason that NaCl and K_2SO_4 have less swelling power because under normal conditions, Na^+ and SO_4 are not preferred.

Now with ATP hydrolysis, the c-value increases at the f^- sites, as a result Na^+, which is not preferred under normal conditions, now becomes much more preferred; as a result, the equilibrium

$$f^- f^+ + Na^+ Cl^- \rightleftharpoons f^- Na^+ + f^+ Cl^- \qquad (5)$$

shifts to the right and cell swelling occurs as a result.

Experimental Testing

1. Testing the dependency of swelling on the concentration of Na^+ in the environment.

Figure 8A shows that mouse kidney tissues exposed to 4° C swells in a normal Ringer Solution. When all the NaCl has been replaced by an isoosmotic concentration of sucrose there was no swelling. Swelling in fact increases quantitatively with increases of the Na^+ concentration in the medium.

2. Testing the role of chloride in cold induced swelling

Figure 8A shows that mouse kidney tissues swell to a much smaller extent in Na_2SO_4 than in NaCl, as the theory has predicted since Cl^- is known to have a stronger binding energy than SO_4^- on ϵ-amino and guanidyl groups (Ling, 1962, p. 173).

3. Testing of the effectiveness of LiCl in promoting cold-injury induced swelling.

As the theoretical curve of Figure 8C shows, Li^+ is another ion which gains in relative adsorption energy as the c-value increases. One could therefore expect it may effectively substitute for Na^+ as indeed it does as shown in Figure 8B. In comparison choline chloride is much less effective.

IV. Cellular Electrical Potentials

A. The Hodgkin-Katz Ionic Theory

There are at least two theories of the cellular resting potential, each one of which is based on a fundamentally different and mutually exclusive theory of the living cell. The Hodgkin-Katz ionic theory of the cellular electrical potential (Hodgkin and Katz, 1949) is based on the view - the membrane-pump theory - that living cells are essentially membrane-enclosed sacs filled with an aqueous solution of proteins, ions, and other small and large molecules. (Pfeffer, 1921; Boyle and Conway, 1941; Dean, 1941) The asymmetrical distribution of solutes across cell membrane is seen as the consequence of the combined action of selective membrane impermeability to some solutes and active pumping of others. (Boyle and Conway, 1941; Dean 1941; Glynn and Karlish, 1975; Ling, Miller and Ochsenfeld, 1973) The electrical potential difference between the intra- and extracellular phases, U, is determined by the concentrations of negatively as well as positively charged ions inside and outside the cell and the permeability of each of these ions through the cell membrane and is quantitatively expressed by the Hodgkin-Katz equation as follows:

$$U = \frac{RT}{F} \ln \frac{P_K [K^+]_{in} + P_{Na} [Na^+]_{in} + P_{Cl} [Cl^-]_{ex}}{P_K [K^+]_{ex} + P_{Na} [Na^+]_{ex} + P_{Cl} [Cl^-]_{in}} \qquad (6)$$

where R and F are the gas and Faraday constants respectively and T is the absolute temperature. P_K, P_{Na}, P_{Cl} are the permeability constants for each of the ions designated. $[K^+]_{in}$, $[Na^+]_{in}$, and $[Cl^-]_{in}$ are the intracellular concentrations of these ions and $[K^+]_{ex}$, $[Na^+]_{ex}$, and $[Cl^-]_{ex}$ are their extracellular concentrations.

In more recent times, the authors of equation 6 suggested a modification of this equation:

$$U = \frac{RT}{F} \ln \frac{[K^+]_{in} + b[Na^+]_{in}}{[K^+]_{ex} + b[Na^+]_{ex}}, \qquad (7)$$

where b stands for P_{Na}/P_K. (Katz, 1966)

B. The Surface Adsorption Theory According to the Association-Induction Hypothesis

An alternative theory of the cellular resting potentials, - the surface adsorption theory - is based on the association-induction hypothesis. (Ling, Miller and Ochsenfeld, 1973; Ling, 1962; Ling, 1969; Ling, 1970)

In the surface adsorption potential theory of the cellular electrical potential, the resting potential arises from the presence of predominantly anionic fixed sites on

the cell surface. Up to now, all evidence suggest that these sites are the β- and γ-carboxyl groups of the cell surface proteins. (Ling, 1962; Ling, 1969; Ling, 1970; Miller, 1977; Ling and Ochsenfeld, 1965) In this theory, the cellular resting potential depends on the nature, the polarity and electronic density or c-value (Ling, Miller and Ochsenfeld, 1973) of these surface anionic sites and the concentrations of external ions which are of such valency and polarity that favor their adsorption on these surface fixed sites. Quantitatively, the resting potential is described by the following equation: (Ling, 1962; Ling, 1960; Ling, 1967; Ling, 1975)

$$U = \text{constant} - \frac{RT}{F} 2n \left(\tilde{K}_K [K^+]_{ex} + \tilde{K}_{Na} [Na^+]_{ex}\right) , \qquad (8)$$

where $\tilde{K}_K$ and $\tilde{K}_{Na}$ are the adsorption constants of K^+ and Na^+ on these surface anionic fixed sites on a microscopically thin layer of the cell surface.

C. Experimental Testing of the Two Theories

The HKI theory demands that the electrical potential depends on the concentrations of intracellular K^+ and Na^+. At least eight independent laboratories have participated in testing this prediction. Their findings were as follows:

1. In 1950 Tobias reported that virtually complete removal of intracellular K^+ from frog muscles after soaking in distilled water did not cause the predicted disappearance of the resting potential. (Tobias, 1950)

2. Grundfest et al, reported in 1954 that injection of high concentration of K^+ into squid axons (1.3 M K-aspartate) did not produce a significant change of the resting potential.(Grundfest, Kao and Altamirano, 1954)

3. In the same Year Falk and Gerard reported a similar failure to observe the expected change of the resting potential following the injection of 3 M KCl or 3 M NaCl into frog muscle fibers. (Falk and Gerard, 1954)

4. In spite of the presence of a normal K^+ and Na^+ concentration gradients across the cell surface of Fundulus eggs, Kao could not detect the presence of a measurable resting potential, (Kao, 1956) even though with a similar technique he and coworkers measured resting potential readily in starfish eggs.(Tyler, Monroy, Kao and Grundfest, 1956)

5. Shaw, et al, (Shaw and Simon, 1955; Shaw, Simon and Johnstone, 1956) found that variation of intracellular K^+ and Na^+ contents did not produce changes of the resting potential of frog muscles according to equation 6.

6. Koketsu and Kimura in 1960 reported normal resting potential in frog muscle cells leached free from most of its K^+ content by prior exposure to a simple half-isotonic sucrose solution. (Koketsu and Kimura, 1960)

7. However, Baker, Hodgkin and Shaw reported in 1961 that perfusion of squid axon freed of axoplasm with NaCl-KCl solution containing K^+ concentration varying from 0 to 600 mM produced a maximum change of potential equal to 60 mV. (Baker, et al, 1961) This finding, however, was in conflict with the observation of no change in the resting potential of similarly perfused squid axon freed of axoplasm reported by Tasaki and Takenaka two years later (Tasaki and Takanaka, 1963) when the perfusing solution of K^+ glutamate was switched to Na^+ glutamate.

The failure to observe a change of U in response to large changes of $[K^+]_{in}$ contradicts the HKI theory. On the other hand, all of these findings, including the conflicting findings of Baker et al, (Baker, Hodgkin and Shaw, 1961) are either in harmony with or are explicable in terms of the association-induction hypothesis as follows:

As indicated in equation 8 the resting potential in this theory does not bear direct relation to the bulk phase K^+ concentration. The increase or decrease of intracellular $[K^+]$ and $[Na^+]$ is not expected to alter U as indeed they were not observed to do so.

D. The Theory of Electrogenic Pump

In the above I have collected some of the evidence pointing to the consistent and repeated observations that the relation between intracellular K^+ concentration and the resting potential predicted by the HKI theory was not observed. I also pointed out how the data supporting the HKI theory as well as those contradicting it agree with the association-induction hypothesis. However, these and similar observations led other physiologists to propose another kind of explanation.

One recalls at the beginning of the 1940's Boyle and Conway applied Donnan's theory of membrane equilibrium to the living cells. At this time, a basically non-energy consuming mechanism was able to explain

(1) Selective K^+ accumulation over Na^+

(2) Cell swelling in KCl but not NaCl

(3) The resting potential

Indeed this was the pinnacle of the membrane theory. The demonstration of Na permeability, led first to the postulation of one ionic pump, the Na pump to explain the selective accumulation of K^+ over Na^+, and then many other pumps. By 1968 some 20 to 30 pumps had been postulated. Yet the Na pump alone under specified and carefully controlled conditions has been shown to consume 15 to 30 times as much energy as the cell commands.

The above cited evidence shows that even by postulating Na^+ and other pumps were not adequate to explain the cellular electrical potentials. To get out of the difficulty, a new kind of pump was postulated called the electrogenic pump.

The membrane potential theory of Ostwald, Bernstein, and Donnan; the ionic theory of Hodgkin-Katz; as well as the sorption adsorption theory according to the association-induction hypothesis all share basic mechanisms with one or another type of potential generating system in the non-living physical world. The electrogenic pump theory, with no known physical model to rely on, is supposedly a potential only generated by vital processes. Indeed its definition is a purely negative statement. Thus Kernan (1970) stated "the electrogenic property of ions may be recognized by a change of membrane potential which cannot be accounted for in terms of passive ion movement and which has some of the characteristics of a metabolic process, such as sensitivity to metabolic inhibitors and a high temperature coefficient. A change of membrane potential which is not accompanied by a significant change in the membrane resistance might also be considered to indicate the presence of electrogenic pumping of ions".

Again it is our opinion that resorting to this semivitalistic interpretation is unnecessary because the surface adsorption theory can offer interpretation which can be readily and quantitatively tested. However, to do so, it also became

clear that the earlier simple equation (Equation 8) describing the potential is no longer adequate; a new equation was introduced. This equation as well as its experimental testing is to be part of the Progress Report.

Appendix

A matrix of chains carrying alternatingly positively charged (P) sites and negatively charged (N) sites is referred to as an NP-NP-NP system. If the chain carrying P sites alternates with vacant (O) sites (sites with no charge) the system is referred to as a PO-PO-PO system. Systems of alternating negative and vacant sites, on the other hand, are called the NO-NO-NO systems.

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ρ_{Na} and H_2O Contents of Various Protein Solutes in the Presence of 1.5M $NaSO_4$

Protein	Time of Incubation (hrs.)	Sample No.	H_2O Content (%)	ρ_{Na}
Lyosozyme	72.5	(4)	82.0 ± 0.085	1.034 ± 0.0054
Y-globulin	72.5	(4)	82.0 ± 0.16	1.035 ± 0.0042
Protamine	72.5	(4)	83.9 ± 0.10	1.021 ± 0.021
Egg albumin	72.5	(4)	82.1 ± 0.058	1.025 ± 0.016
Bovine serum albumin	72.5	(4)	81.9 ± 0.063	1.003 ± 0.0048
β-lactoglobulin	72.5	(4)	82.6 ± 0.029	1.016 ± 0.0052
Pepsin	91	(4)	83.4 ± 0.11	1.052 ± 0.006
Chymotrypsinogen	91	(4)	82.7 ± 0.089	1.024 ± 0.0095
Chondroitin sulfate	91	(4)	84.2 ± 0.061	1.030 ± 0.0033
Fibrinogen	70	(4)	82.8 ± 0.12	1.024 ± 0.002
Hemoglobin	70	(4)	73.7 ± 0.073	0.942 ± 0.006
Y-globulin (human)	70	(4)	83.5 ± 0.16	1.037 ± 0.005
Ribonuclease	70	(4)	79.9 ± 0.19	1.004 ± 0.006

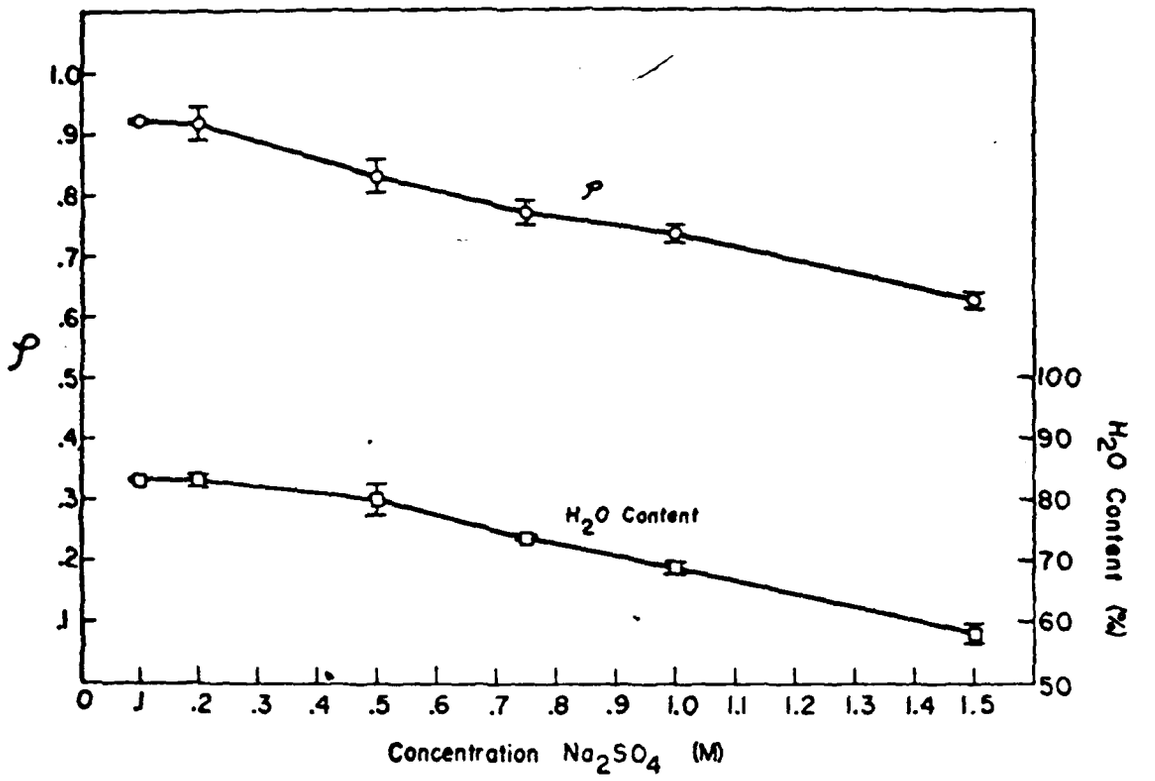
TABLE 1

	Osmolarity of Bathing Solution (mOSM)	$\frac{\text{Final wt.}}{\text{Initial wt.}} \times 100$ (%)	Schwellingswasser (%)
1	56.4	2.37 ± .09 (4)	46.7 ± 3.23
2	78.9	2.11 ± .08 (4)	29.7 ± 4.50
3	101.4	1.72 ± .07 (4)	30.2 ± 6.20
4	123.8	1.43 ± .03 (4)	39.6 ± 5.17
5	146.2	1.20 ± .05 (4)	56.5 ± 9.84
6	168.6	1.15 ± .02 (4)	46.8 ± 6.04
7	191.2	1.16 ± .07 (4)	46.0*
8	213.6	1.00 ± .04 (4)	43.4 ± 13.6
9	236	0.99 ± .02 (4)	

* Average of 3 values.

TABLE 2

The percentage of Schwellingswasser or non-water in frog muscles equilibrated in hypo- and isotonic Ringer-GIB medium



The ϕ value of sodium ion and water contents of gelatin after equilibration in solutions of sodium sulfate at different concentrations (abscissa). Note that at 1.0 M Na_2SO_4 the ϕ value is considerably lower than unity indicating a substantial portion of the water here is unaccessible to sodium sulfate.

FIGURE 1

Pennsylvania Hospital
Philadelphia, Pennsylvania 19107

CONTINGENT FEE

(a) He ☐ has, ☒ has not, employed or retained any company or persons (other than a full-time bona fide employee working solely for the offeror) to solicit or secure this contract, and (b) he ☐ has, ☒ has not, paid or agreed to pay any company or person (other than a full-time bona fide employee working solely for the offeror) any fee, commission, percentage, or brokerage fee contingent upon or resulting from the award of this contract; and agrees to furnish information relating to (a) and (b) above, as requested by the Contracting Officer. (Interpretation of the representation, including the term "bona fide employee", see Code of Federal Regulations, Title 41, Subpart -1.5).

EQUAL OPPORTUNITY

(a) He ☒ has, ☐ has not, participated in a previous contract or subcontract subject either to the Equal Opportunity clause herein or the clause originally contained in section 301 of Executive Order No. 10925, or the clause contained in Section 201 of Executive Order No. 11114; that he ☒ has, ☐ has not, filed all required compliance reports; and that representations indicating submission of required compliance reports, signed by proposed subcontractors, will be obtained prior to subcontract awards. (The above representation need not be submitted in connection with contracts or subcontracts which are exempt from the equal opportunity clause.)

(b) The bidder (or offeror) represents that (1) he ☒ has developed and has on file, ☐ has not developed and does not have on file, at each establishment affirmative action programs as required by the rules and regulations of the Secretary of Labor (41 CFR 60-1 and 60-2) or (2) he ☐ has not previously had contracts subject to the written affirmative action programs requirements of the rules and regulations of the Secretary of Labor. (The above representation shall be completed by each bidder (or offeror) whose bid (offer) is $50,000 or more and who has 50 or more employees)

STATEMENT REGARDING ACQUISITION OF FACILITIES

The contractor, represented by an executive corporate official, or his equivalent in non-corporate entities, either expresses in writing his unwillingness or financial inability to acquire the necessary facilities with his resources.

Harry E. Heston (date)
Vice President
(Official Authorized to Sign for Institution)

PENNSYLVANIA HOSPITAL
The Nation's First Hospital · Founded 1751

DEPARTMENT FOR SICK AND INJURED
EIGHTH AND SPRUCE STREETS
PHILADELPHIA, PENNSYLVANIA 19107
TELEPHONE (215) 829/

November 10, 1980

Mrs. Farrington
Office of Naval Research
Code 613 KF
800 N. Quincy St.
Arlington, VA 22217

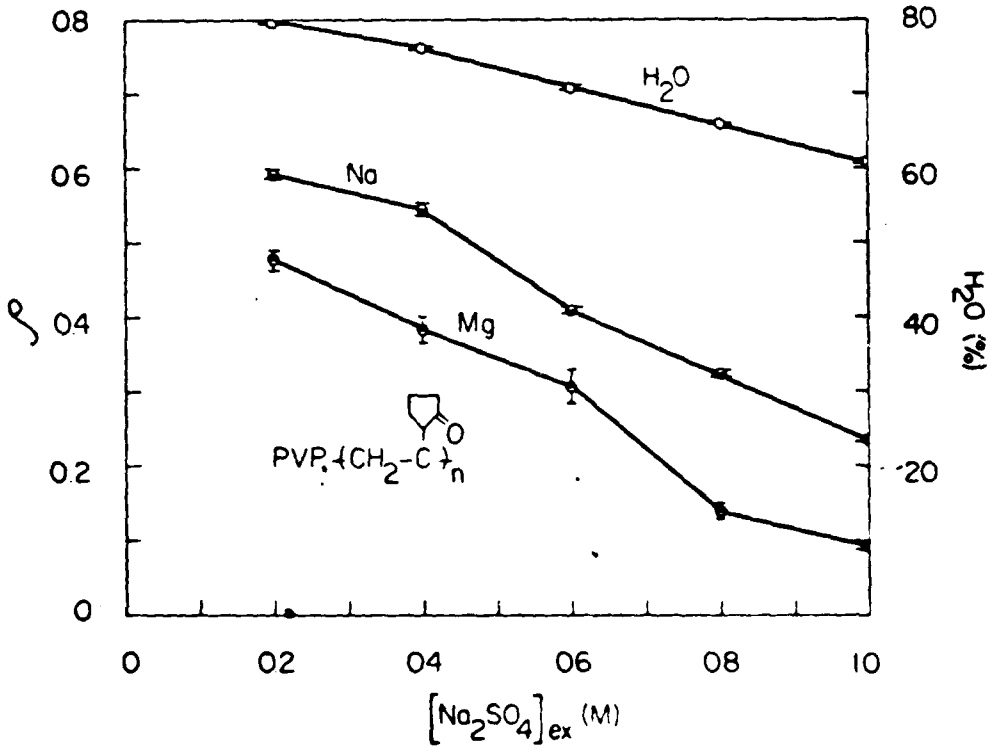
Dear Mrs. Farrington,

The hospital is in a circumstance where it is not in a position to pay for equipment used for research in Contract # N00014-79-C-0126. Without this equipment the project cannot be continued as orignally awarded. Please accept this as a poverty statement for Pennsylvania Hospital.

Sincerely,

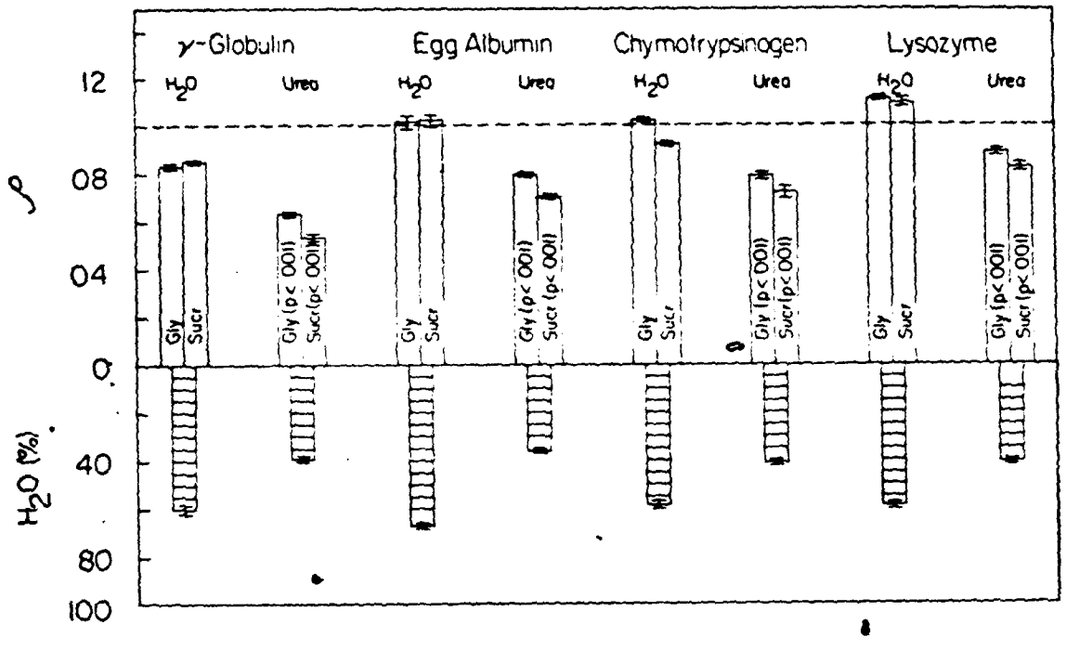
Harry Heston
Vice President

HH/db



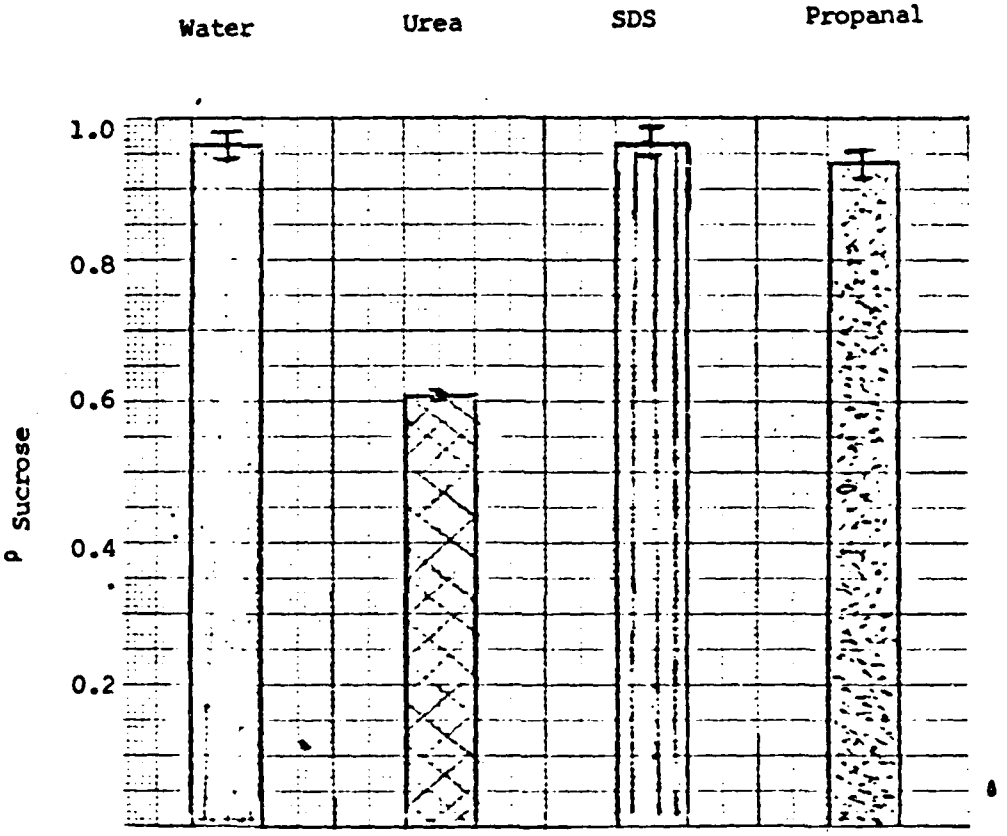
The ρ value for sodium and magnesium in a poly vinylpyrrolodine (PVP)-water system in varying concentrations of sodium sulfate. Note the very low ρ value at the higher salt concentrations. Data again indicate major portion of the water being unaccessible to magnesium and to sodium.

FIGURE 2



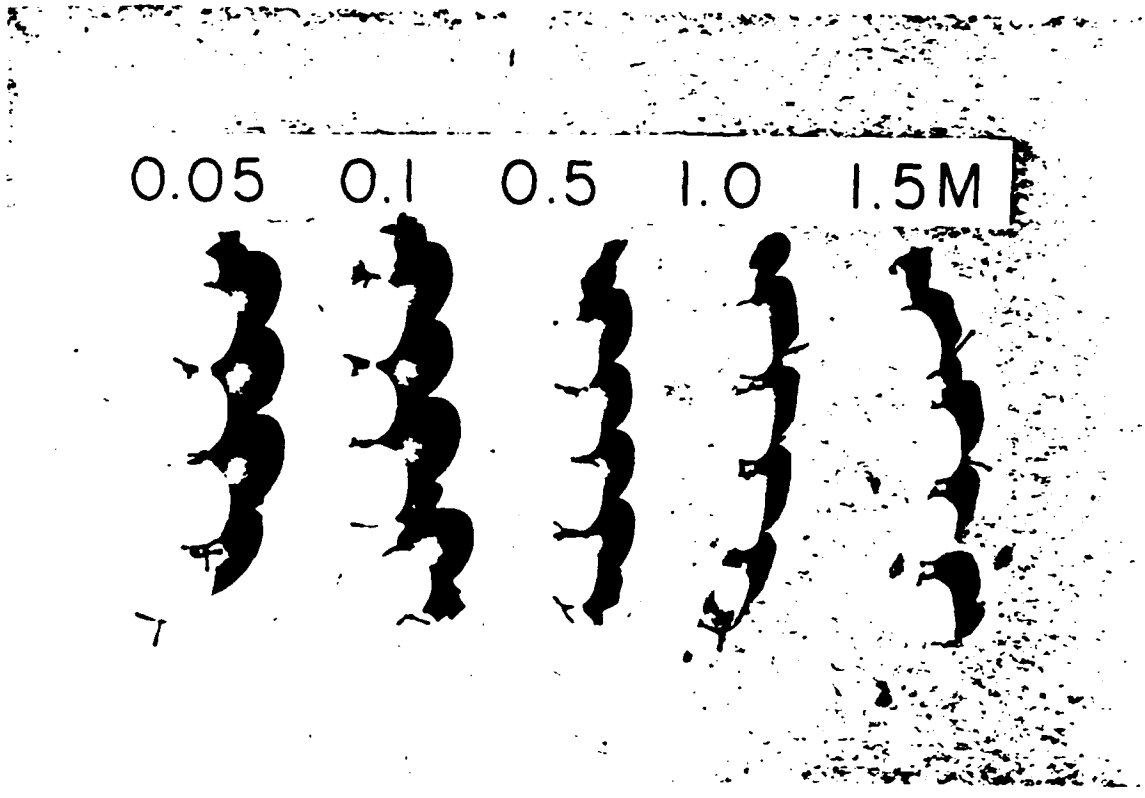
The change of ρ value for sucrose and glycine of various pure proteins in response to interaction with urea (10 M). Data indicated decrease of ρ value for the probe molecules glycine and sucrose in consequence of the interaction of urea with protein. It is well known that urea unravels the intramolecular hydrogen bonding creating random coils with NHCO groups directly exposed to bulk phase water.

FIGURE 3



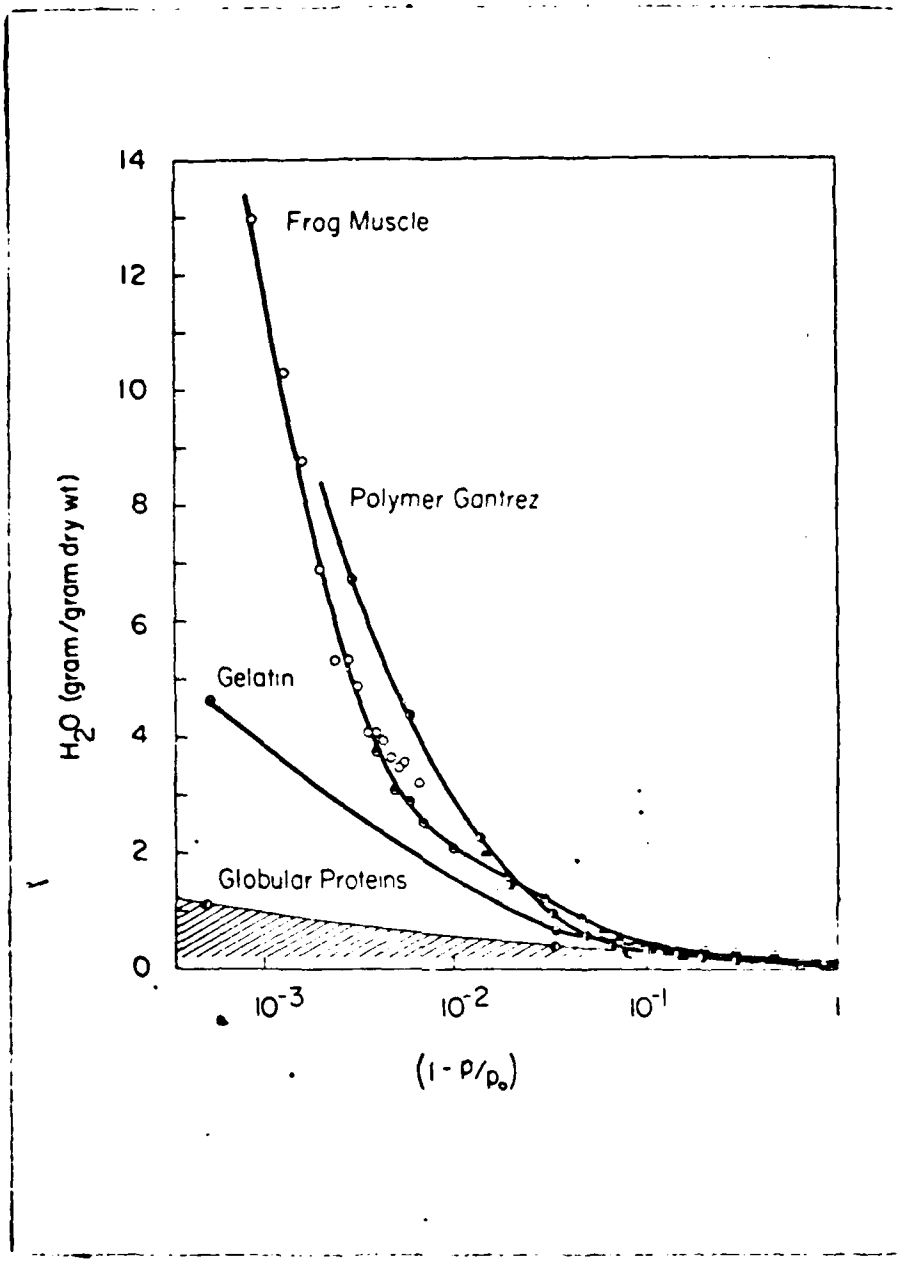
Data indicate a decreasing ρ value in consequence of interaction of a native protein with urea (10 M). The contrast to other denaturants sodium dodecyl sulfate (SDS) and n-propanal both are known to dissociate only tertiary structures but are known also not to unravel α helical secondary structures. Indeed SDS is known to promote the formation of more α helical structures.

FIGURE 4



[illegible] and shrinkage of a non charged polymer water system in the presence of [illegible] concentrations of sodium citrate. Note that at equilibrium the bags [illegible] initially similar concentration of the polymer, swelled in dilute salt solution but shrank in the more concentrated salt solution. Dialysis tubing used is readily permeable to sodium citrate and are used only to hold the polymers. The data indicate that a polymer water system can shrink and swell in the same manner as the living cells when they are placed in hyper- or hypotonic solutions. No semi-permeable membrane is needed. All that is required is water polarized and thus having a low q value.

FIGURE 5



Adsorption of water by living cells and model systems in environments containing water at different activities. The uptake of water expressed as H_2O (gram/gram dry wt) is plotted against $1 - P/P_o$. As explained in the text abscissa is essentially equivalent to the osmotic pressure. The frog muscle curve consists of two parts: the part to the left with empty O's was obtained by immersing muscles in isotonic and hypotonic solutions; points represented by half shaded O's were obtained by vapor equilibrium and were taken from Ling and Negendank. Water uptake by the polymer Gantrez was obtained by equilibrating Gantrez water in cellophane bags in solutions of sodium citrate of different strength. Shaded area labelled Globular Proteins was derived from the data in the literature and so was the data for gelatin.

FIGURE 6

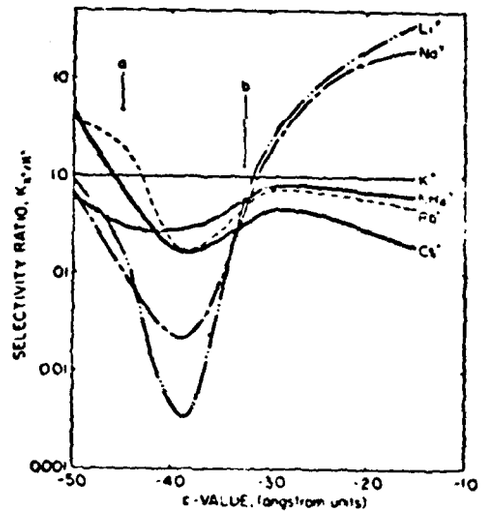
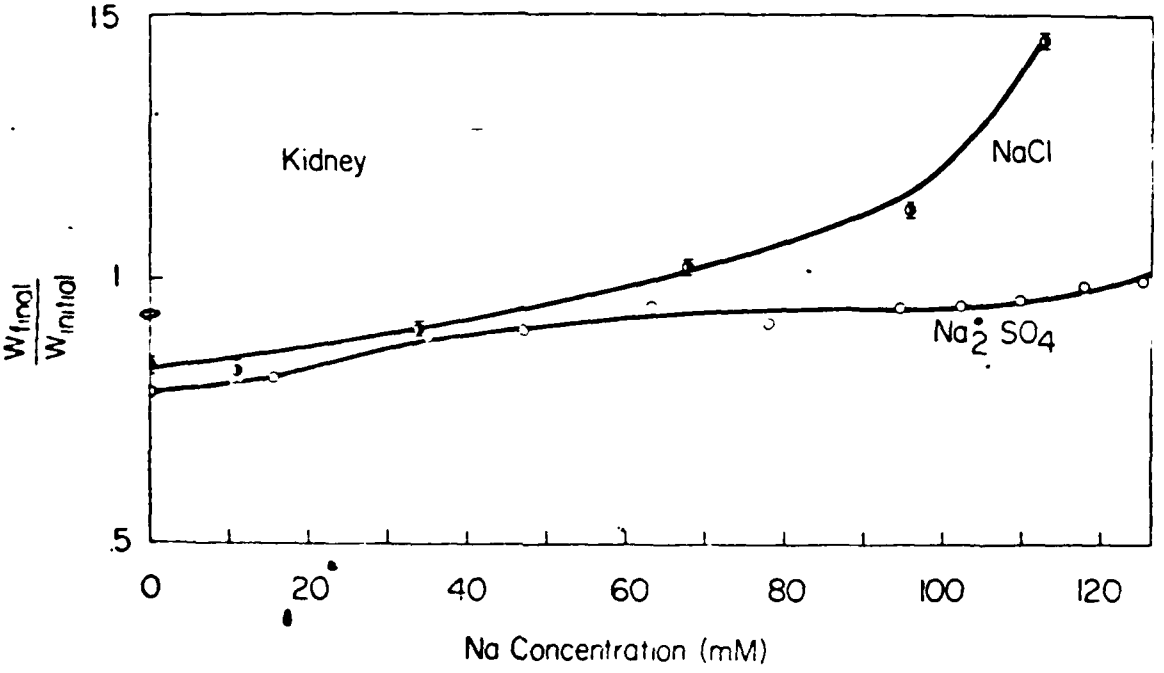


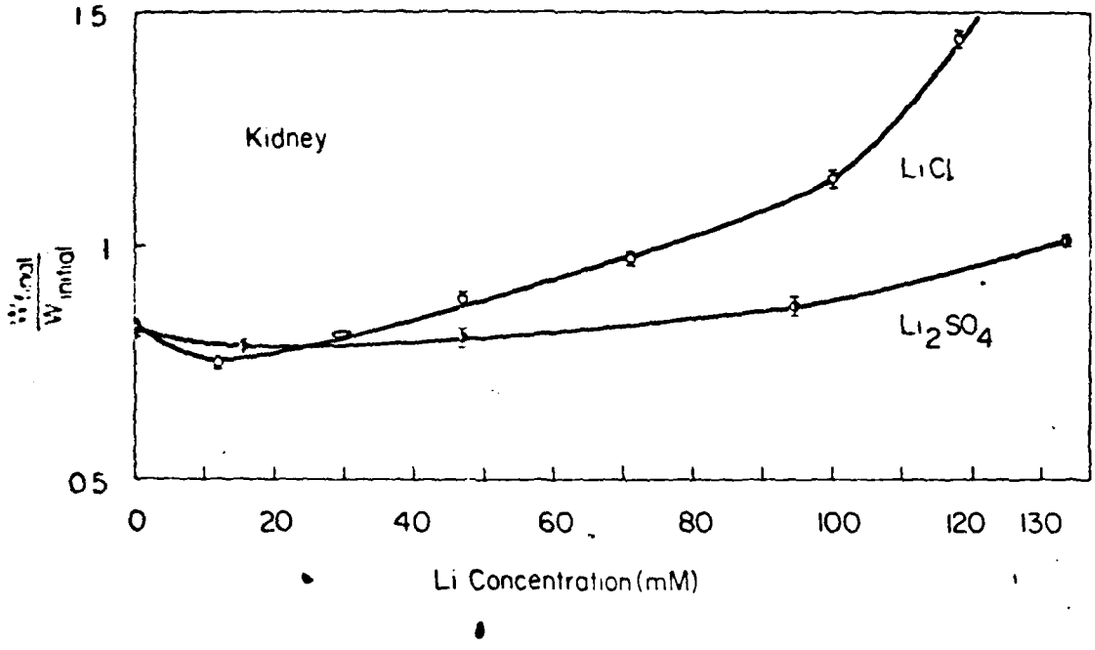
Figure 8. The relation between the selectivity ratios of various cations and the ε-value. The K^+ ion is taken as unity and selectivity ratios are calculated from the association energies given in Figure 4.11 of Ref. 7, (polarizability of anionic group: 2.0×10^{-24} cm^3) divided by 2 (see Sec. 4, 4A of Ref. 7). These curves are theoretical.

FIGURE 7



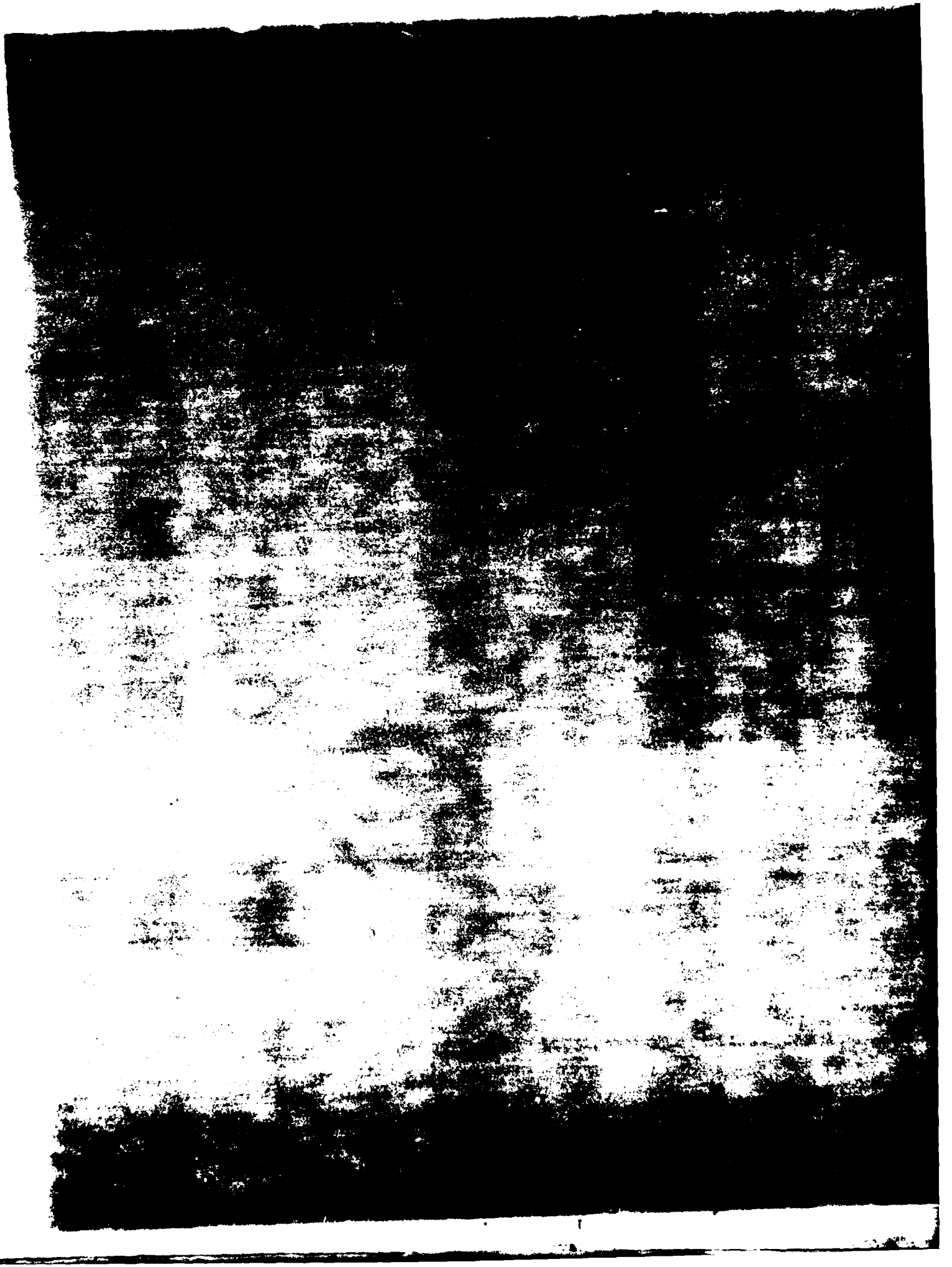
Swelling of cold injured rat kidney occurs only in the presence of sodium chloride but not in the presence of equieosmotic concentrations of sucrose. Indeed data show that the swelling effect is specific both to the sodium and to the chloride ion thus sodium sulfate had much less swelling effect.

FIGURE 8A



Swelling and shrinkage of cold injured rat kidney in the presence of lithium chloride. Data indicate lithium chloride also has an effect in promoting swelling and that like the case of sodium salt the sulfate salt is less effective.

FIGURE 8B



Progress Report

For Part of the Work Accomplished Between
Sept 1, 1978 - to Aug 31, 1979
under ONR Contract N00014-79-C-0126

Principal Investigator:

Gilbert N. Ling, Ph.D.
Director, Department of
Molecular Biology
Pennsylvania Hospital
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Project I. NMR Relaxation Times of Water in the State of Polarized Multilayers

In 1965 Ling presented the polarized multilayer theory of cell water as part of the association-induction hypothesis. In years following, NMR methods were brought in to verify the concept that cell water exists in a different state than normal liquid water. It was primarily through NMR, that Hazlewood, Cope and Damadian entered the cell water investigation. In many ways these researchers have been highly productive. Nevertheless, NMR did not produce an unequivocal proof. More than one reason exists for this uncertainty; not the least, is that up to now water existing in the state of polarized multilayers was largely a matter of an as yet unproven scientific conjecture. Clearly if we do not know in the first place that a certain kind of water exists anywhere, it would be impossible to prove that it exists in living cells. It is now possible for us to report that work supported by ONR and NIH has altered this state of affairs.

(1) Water in the State of Polarized Multilayers

One of the major experimental achievements of this laboratory in recent years is our success in confirming the theory of polarized multilayers: all one requires is a matrix of linear chains carrying oxygen atoms at intervals of space roughly equal to the diameters of two water molecules. Water molecules distributed among these chains do indeed exhibit reduced solubility for Na^+, sugar and amino acids as predicted theoretically and from the (apparent) equilibrium distribution coefficient, or P-value of these probe molecules, one can calculate the minimal number of water molecules that are affected by each of the oxygen atoms. This number (14 to 20)is so large that they cannot exist as a single layer. In other words multilayers of water molecules are affected. (Page 8, line 10 of MS #1) "Years ago, Brunauer, Emmett and Teller (36) showed that charged sites can influence more than one layer of adsorbed molecules only if these molecules possess a large permanent dipole moment, as it is the case with water ($\mu = 1.834 \times 10^{-18}$ e.s.u.). In other words, oxygen atoms in PEO and other polymers can influence distant water molecules only by a mechanism of propagated electrical polarization involving both induced and permanent dipole moments of the 'target' as well as intervening water molecules. This is just another way of saying that these water molecules exist in the state of polarized multilayers."

With a collection of the water polarizing polymers of diverse structure on hand, we undertook a painstaking task of purifying and removing (paramagnetic)

impurities. We then studied the NMR relaxation times of the water in these polymer-water systems. The results are illustrated in Figure 1.

Much important information can be derived from these data. Only two facts need be mentioned.

1. At a water content that corresponds to a low P-value for Na, sucrose, etc. the T_1 value of the water proton is indeed considerably reduced (i.e., 500 msec vs. 3000 msec for pure liquid water).

2. T_1 and T_2 in this range of water content are remarkably close, in sharp contrast to the large differences between T_1 and T_2 seen in almost all living cells (T_1/T_2 = 10 in muscle).

The equality of T_1 and T_2 show that the bulk of polarized water has a correlation time, probably close to 10^{-11} sec compared to that of normal liquid water at 3×10^{-12} sec.

(2) NMR Relaxation Times of Water in Living Tissues

In recent years both Hollis' group in Baltimore and Pintar's group in Waterloo, Canada, came to the conclusion that the difference in T_1's of living tissues is simply the result of the differences in the total water contents. Although a number of scientists including Beall, et al (Physiol. Chem. Phys. 8:281, 1976), Kagimoto, et al (J. Natl. Cancer Inst. 59:335, 1977) produced evidence to the contrary this idea is still believed by many. Two of the most persuasive arguments are that (1) the T_1 vs. H_2O content plot of many types of tissues can be plotted and shown to fall on a single line and (2) that although normal spleen and kidney of the mouse have different T_1 values, if their water contents were varied by exposure to solutions containing varying concentrations of NaCl, the T_1 vs. H_2O content plot again falls on the same line.

Our own data illustrated in Figure 2 completely contradict this view. It shows that if the water contents of different tissues were varied by changing the osmolarity of the external solution, the T_1 vs. H_2O content plots remain separate. From these and similar studies we reached the conclusion that while water content undoubtedly affects T_1, its effect is minor (ca. 10%) in the total difference of T_1 between different tissues.

Project II. Is the Theory of Donnan Membrane Equilibrium Valid?

A physico-chemical theory that has served as a pillar of the membrane theory was presented by F. Donnan in the early part of the century. This theory deals with the equilibrium distribution of permeant ions between two solution phases separated by a membrane which is impermeable to one species of the ions present in one of the solution phases. If one designates the side containing the impermeant ion (say an anion) as the inside phase and the other side as the outside phase, Donnan's theory can be represented by a set of simple equations:

$$\left[\frac{(C_i)_i}{(C_i)_o}\right]^{1/n} = \left[\frac{(a_j)_o}{(a_j)_i}\right]^{1/m} = r \qquad (1)$$

where n and m are the valencies of the ith cation C_i and that of the jth anion a_j. r is called the Donnan ratio. Furthermore, there should be an electrical potential difference between the two phases, or the membrane potential Ψ

$$\Psi = \frac{RT}{F} \ln r, \qquad (2)$$

where R, F are the gas and Faraday constant, T the absolute temperature. It is clear that this set of equations prescribes a finite mutually dependent relation between all permeant ion distribution and the potential. Change of the concentration of one ion species, for example, would affect the distribution of all others as well as the potential.

Our own investigation of the Donnan theory of membrane equilibrium was prompted by the following:

(1) Our investigations of ionic distribution patterns in living cells do not indicate obedience to the predictions of the Donnan theory. Studies with the aid of the effectively membrane-less open ended cell (EMOC) preparation also showed that ion distribution patterns are not governed by membrane pumps.

(2) At the time Donnan proposed his theory, there was no adequate technology to fully test the prediction of this theory. Advances in recent years have made available these technical means for investigation.

Basically we studied the distribution of a variety of mono-, di, and trivalent ions across collodion membranes which enclose a solute containing an impermeant charged polymer with large molecular weight and rather high density of negative charges (linear chains of polysterene sulfonate).

The data of Table 1 deals with 9 ions all isotopically labelled. The establishment of equilibrium was proven by the near identity of values of distribution ratio under all four columns with different lengths of time of incubation (I, II, 2 weeks; III, IV, 49 and 97 days respectively) and to different sides of the membrane where the isotopes were added (I, II, added to the inside of the bag; III, IV, added to the outside of the bag).

The data represents the ratio $\frac{(a_H^i/a_{H^+}^o)^{Z_i}}{(Ci)_i/(Ci)_o}$ when a_H^i and $a_{H^+}^o$ are the activities of protons measured with a pH meter at the end of incubation and Z_i is the valency of the ith ion under study. $(Ci)_i$ and $(Ci)_o$ are the internal and external concentrations of the ith cation. The Donnan theory predicts that this ratio should always be unity.

Our data show that for all 9 ions studied none shows a ratio of unity.

The results given above show that the overall pattern of ionic distribution does not follow the prediction of Donnan's theory of membrane equilibrium, according to which ions are entirely free inside and outside of the semipermeable membranes.

We believe that a serious error was made in assuming that ion adsorption does not take place or takes place but to a negligible degree. Unfortunately at the time Donnan' theory was proposed, no careful study was made to test if this simplifying assumption was valid. However, half a century later, we now have technology that permits rigorous testing, i.e., ion specific electrodes which can measure ionic activity of only free ions. By comparing the ion activity measured with the total ion concentration determined with atomic absorption methods, for example, one can readily deduce how much of the ion in the cellophane bag was free and how much adsorbed. It turned out a sizable fraction of all ions studied were adsorbed. This adsorption fraction could amount to 95% of the total Cu^{++} in the dialysis bags, for example.

The next question is, "Accepting the adsorption of a major portion of the cations, how do the free ions distribute across cellophane membrane?" In other words, "Do the free ions follow the theory of Donnan equilibrium and obey the prediction of Equation 1 and 2?"

To test this we employed the highly ion specific solid state electrodes from Orion, Inc. With these electrodes we were able to measure the activity of H^+ and of divalent Cu^{++} and Cd^{++} inside and outside the same bag. After making

absolutely certain equilibrium was reached, these activities were recorded. From the H^+ ratio between the inside and the outside phase of the bag, one can calculate the ratio of the divalent Cu^{++} according to Equation 1 on the basis of the measured H^+ activity ratios. These predicted values of Cu^{++} distribution on the basis of the Donnan theory were then compared with the Cu^{++} or Cd^{++} ratios actually measured. Table 2 presents one such series of studies. The polymer is polystyrene sulfonate (M.W. 500,000). The discrepancy between theory and experimental values is so great that there is little question that the Donnan theory is incorrect.

An alternative theory was also proposed, which predicts ratios quite close to those observed. Indeed this was a variant of the general equation for solute distribution in living cells we proposed in 1955. But to avoid a too lengthy report, its details will not be given here.

Project III. Mechanism of Cell Swelling

In the "Background", we have briefly described the failure of the membrane theory to account for cell swelling which can occur in hypotonic solution or solution containing high concentration of KCl with or without an intact cell membrane. We have also presented earlier work accomplished under ONR supported contracts, that there are fundamental similarities between swelling induced in high concentration of KCl (100 mM) of normal cells, and swelling induced by injury which depends on the presence of a high Na^+ concentration in normal Ringer Solution (100 mM). The basic mechanism is as follows: One of the major forces holding together the cell proteins are salt linkages between the negatively charged β- and γ-carboxyl groups and positively charged ϵ-amino groups and guanidyl groups. In normal resting cells, the electron density or c-values of the β- and γ-carboxyl groups are such that they prefer both K^+ and fixed amino groups over Na^+ as according to theoretical calculations published in 1962. Since in normal plasma or Ringer Solution, the K^+ concentration is low, normal cells in these media remain unswollen. However increases in the external K^+ concentration dissociates the salt linkages. As a result more water is taken up and the cell swells. This concept has been rigorously discussed in a paper that Ling and Peterson published in 1977 and was shown able to account for the complex swelling behaviors not only to KCl but to high concentrations of a variety of other salts.

Another crucial test of this theory involves the predicted concomittant binding of chloride ion in the cell with swelling. Thus if we represent β- and γ-carboxyl groups as f^- and the ϵ-amino and guanidyl groups as f^+, the basic salt-

linkage dissociating reaction in swelling is

$$f^+ f^- + K^+ Cl^- \longrightarrow f^- K^+ + f^+ Cl^- \quad , \qquad (3)$$

This equation predicts the creation of new adsorbed Cl^- with increasing KCl concentration. The swelling of frog muscle in increasing concentrations of KCl thus include two distinct steps: salt linkage dissociation and further multilayer adsorption of H_2O. Indeed to account for the swelling quantitatively, we have presented theoretical curves that appeared in Science in 1977 (Figure 2A). These theoretical curves are composed of three distinct fractions shown in the inset of Figure 14 (also Figure 2A, bottom of left figure).

To test this prediction of Cl^- binding with swelling we relied on a basic method we have evolved. The Cl^- efflux of a normal frog muscle in a normal Ringer Solution is extremely rapid. This reflects the rapid exchange of free chloride ion between the inside and outside of the cell, as shown in Figure 3 where the solid curve line represents the efflux after correction for contribution from connective tissues. Most of the labelled intracellular Cl^- (about 5 mM) and Cl^- in the extracellular space (8 to 10mM) is fully washed out within 20 minutes. A very slow exchanging fraction amounts to less than 0.1 mM and is thus quantitatively negligible. Thus the normal Cl^- efflux curve as shown in Figure 3 includes the Cl from the extracellular space (fraction a, not sorted out) and intracellular free Cl^- (fraction b).

Incubation in a Ringer Solution containing 30 mM KCl causes some swelling. As predicted, a new slowly-exchanging fraction of Cl^- makes its appearance (Figure 4). This fraction (fraction c) is quite distinct from both the a and b fractions and the small very slowly exchanging fraction mentioned above. Note that the magnitude of this new fraction, indicated by the intercept on the ordinate of the new c-fraction is from 3 to 6 mM.

Further increase of KCl to 50 mM produced little change of the c-fraction intercepts (Figure 5). One is reminded of the fact that a shoulder at 50 mM was also observed in swelling.

Further increase of KCl to 75 mM caused another significant increase of the c-fraction to from 8 to 12 mM corresponding once more to increased swelling (Figure 6).

Further increase of KCl to 100 mM caused the c-fraction to increase still further to from 12 to 30 mM (Figure 7). At 140 mM KCl, c-fraction reached the value of 40 to 50 mM and at KCl = 200 mM, the c-fraction reached a maximum value of 50 to 60 mM (Figure 8) which stayed put at 250 mM.

From 140 mM KCl on, a new fraction occasionally appeared, which in time constant is different from a, b, and c, as well as the original very slowly exchanging

fraction (Figure 8). At KCl = 250 mM, the new d-fraction began to dominate until at 350 mM KCl, a still slower e-fraction appeared (Figures 9 and 10).

Figures 3 to 12 each includes three sets of data. Actually many more than three sets were run at each concentration. The overall average and its standard error of each of the fractions or sum of two (a and b, d and e) are shown separately in Figure 13, and together in Figure 14.

The three of experimentally derived curves as well as their total are to be compared with the sets of theoretical curves which we had derived entirely on the basis of the theory.(inset of Fig 14) There are, to be sure, some quantitative differences. But there is little doubt that the two swelling steps, one occurring between 30 mM to 100 mM, and the other occurring between 200 and 300 mM, both have a corresponding observed new bound Cl^- fraction, as to be expected from Equation 3. The only significant departure is that the c-fraction does not remain constant but actually disappeared, apparently to re-emerge as part of the d and e fraction at high KCl concentrations.

In summary, this near total confirmation of a theoretical prediction of the theory of KCl induced swelling with new findings otherwise unknown till now, has been one of our most satisfactory experiences in the recent past.

Project IV. A More General Equation of the Cellular Resting Potential and Its Experimental Verification

In the "Background" a bird's eye view was presented concerning the extensive experimental evidence against the Hodgkin-Katz (HKI) theory of cellular potential, of the emergence of two new theories: the electrogenic pump (EP) theory, which is another evolutionary phase of the membrane-pump theory, and the surface adsorption (SA) theory as part of the association-induction hypothesis.

Both the HKI and SA theories are quantitative theories exactly formulated. Such is not the case with the electrogenic pump theory, which remains at a qualitative conjectural stage.

A new challenge to all theories came from the finding that cells loaded with Na^+ and depleted of K^+ by equilibration in a Ringer Solution containing little or no K^+, regained its resting potential soon after exposure to a normal Ringer Solution containing its usual normal concentration of K^+. Analysis of the total K^+ and

Na^+ content showed that the ionic gradient would predict a much lower potential. The excess potential observed was explained by an electrogenic pump. This concept is vitalistic since, there is no known physical model of this kind; it also violates the law of conservation of energy to postulate still more pumps.

In contrast, by combining two well known "components" of the association-induction hypothesis, we derived a new equation for the resting potential (Ling, 1979). This equation incorporates both surface anionic sites as the seat of the potential and adds that there is site-to-site near neighbor interaction, or auto-cooperativity among the sites:

$$\Psi = \text{constant} - \frac{RT}{F} ln \frac{1}{[K]_{ex}} \left[1 + \frac{\xi - 1}{\sqrt{(\xi - 1)^2 + 4\xi \exp (Y/RT)}} \right], \qquad (4)$$

where $- Y/2$ is the nearest neighbor interaction energy and

$$\xi = \frac{[K^+]_{ex}}{[Na^+]_{ex}} \cdot K^{oo}_{Na \to K} \qquad (5$$

$K^{oo}_{Na \to K}$ is the intrinsic equilibrium constant of the Na^+ to K^+ exchange.

Equation 4 predicts behavior of the resting potential at low external K^+ which neither the HKI theory nor our own earlier version of the surface adsorption theory do. Two summer students supported by ONR funds have already completed two papers successfully testing this Equation 4. There is no space to recount all of these findings here. Only one additional figure is presented as shown as Figure 15. The inset is a set of theoretical curves of Ψ at varying values of $[K^+]_{ex}/[Na^+]_{ex}$ and θ, where $\theta = \exp (Y/RT)$. Note that a θ decrease means increase of the nearest neighbor interaction energy. The main curves of Figure 15 represent two sets of resting potential measurement in frog sartorius muscle with Ling-Gerard microelectrode. The theoretical curves corresponding to the experimental parts yield $K^{oo}_{Na \to K}$ of about 100 and $- Y/2$ of around 1.0 Kcal/moles.

The drop of potential at very low K^+, first observed by S. Weideman confirmed by Ruzyllo and Vick in cardiac muscle tissues are thus quantitatively explained by the surface adsorption theory of the AI hypothesis. Neither the HKI nor the electrogenic pump theory can do the same.

Progress Report on Special Projects

(1) The Video Tape Project

This project has been steadily progressing. Many tapes have been taken. Since we have become increasingly aware on one hand of the great potential of this approach, we are also aware of the extreme brevity of a 30-minute tape in which to transmit some very complicated information. Hence our reluctance to make a quick finish. But at no time, have we any doubt that a tape will be finished that is worthy of the time as well as the financial investment made by ONR ($3,000). Incidentally, the Zahn brothers (New York) who have been doing this, have told me that they have long used up the allotted fund but have become deeply involved and interested in the project and have not allowed this to deter them from the goal.

(2) The Summer Student Program

A combination of reasons has made the summer student program a critical component of our department and ONR has played a major role in this for many years.

You are undoubtedly aware of Dr. William Negendank, who has among other things arranged the recent grantee meeting at Philadelphia. Dr. Negendank began his scientific research career as a participant of my summer student program.

The association-induction hypothesis has developed to a very productive stage. Yet my limited staff cannot fully exploit all these great opportunities since each member of my staff is already immersed in her enterprise.

Of course, one can ask, "Why do you have to do all of these? Why can't you let somebody else outside your immediate sphere do them?" My answer to this, in part, is we have accumulated considerable techniques and know-how that it would be most efficient to work directly with us to start them off.

May I also add that as far as ONR cost-efficiency is concerned, how else can you get first rate people to do first rate work at the very low rate of $1000 for three months, or an equivalent of $4000 per year?

Finally, what have my summer students done in the summer of 1973? Before presenting the details, I want to mention that due to rampant inflation and astronomical cost increase of laboratory supplies, we had to use part of the $10,000 given for summer students to provide the supplies for their work. Only five students were employed.

(1) John Baxter (Boudoin) in a total of two months time completed a microelectrode study of the resting potential of frog sartorius muscle in response to ouabain,

and achieved complete confirmation of the theoretical prediction of the surface-adsorption theory under the influence of cardinal adsorbents. Figures A and B show a comparison of the theoretical curves with those actually observed. John's work opened the way for future study of the cellular electrical potential under all kinds of pharmaceutical and physiological agencies - which up to this moment has not been more productive than postulating more pumps (electrogenic). John's work will be written up as a full-length paper very soon.

(2) Mark Ling (Harvard, Duke University Medical School) provided the work that has opened the door to another major avenue of research - identification of cell proteins that are responsible for water polarization and K adsorption. His work led to evolvement of new electrophoretic technique for tracking down the responsible proteins by progressive lysing of the human red cells.

(3) Karen Holmes (Yale University) continued another project that was initiated by another summer student some years ago. The effect of external K^+ concentration on the oxygen consumption rate. The bell-shaped curve, as shown in Figure C, to the best of my knowledge, has never been demonstrated before. The great significance lies in the fact that the mitochondria are within the cell and according to the conventional membrane theory, already bathed in 100 mM K^+. Why should a further increase of 10 mM K^+ outside the cell make such a difference? On the other hand, the data offer powerful support for the association-induction hypothesis and opens up new ways to study K^+ mediated respiration control.

(4) Gordon Lindberg (Haverford College) studied rat liver mitochondria which were shown to behave essentially like muscle cells and not at all like that postulated by Peter Mitchell and others. The mitochondria membrane appears quite permeable to water, K^+, Na^+ as well as other solutes and valinomycin does not act as specific K^+ ionophore but like ouabain and ATP acts as cardinal adsorbents. Thus valinomycin appears to change $K^{oo}_{Na \to K}$ like ouabain, and ATP increases total fixed anionic sites available for alkali-metal ion adsorption presumably by salt-linkage dissociation.

(5) Chet Kwon (Univ. of Penna.) continued study of injury induced brain swelling. He extended the osmotic pressure vs. water sorption data of living tissues by vapor equilibrium methods. Further investigated NaCl dependent cold-induced swelling of mammalian tissues. It was his work as a summer student two years ago that produced the two figures (Figures 8a and 8b) cited in the Background Information.

Publications

Published

G. N. Ling - "Maintenance of Low Sodium and High Potassium Levels in Resting Muscle Cells", J. Physiol. 280:105-123 (1979)

G. N. Ling - "Two Opposing Theories of the Cellular Electrical Potential: A Quarter of a Century of Experimental Testing", Bioelectrochem. and Bioenergetics 5:411-419 (1978)

G. N. Ling, J. M. Ochsenfeld, C. Walton and T. J. Bersinger - "Experimental Confirmation, from Model Studies of a Key Prediction of the Polarized Multilayer Theory of Cell Water", Physiol. Chem. Phys. 10:87-88 (1978)

G. N. Ling - "Experimental Confirmation of a Key Prediction of the Polarized Multilayer Theory of Cell Water from Model Studies", 6th Int. Biophys. Cong., Kyoto, Japan, Sept. 3-9 (1978) pp. 389

G. N. Ling - "How Does Reduced External K^+ Concentration Affect the Rate of Na^+ Efflux? Evidence Against the K-Na Coupled Pump but in Support of the Association-Induction Hypothesis", Physiol. Chem. Phys. 10:353-365 (1978)

G. N. Ling - "The Polarized Multilayer Theory of Cell Water and Other Facets of the Association-Induction Hypothesis Concerning the Distribution of Ions and Other Solutes in Living Cells", in The Aqueous Cytoplasm, pp. 23-60, Ed. Alec D. Keith, Marcel Dekker, Inc. NY (1979)

G. N. Ling - "The Polarized Multilayer Theory of Cell Water According to the Association-Induction Hypothesis", in Cell-Associated Water, Ed. Walter Drost-Hanson & James Clegg, Academic Press, Inc. NY pp. 261-270 (1979)

G. N. Ling - "The Equations for Cellular Resting Potentials According to the Surface Adsorption Theory, A Corollary of the Association-Induction Hypothesis", Physiol. Chem. Phys. 11:59-64 (1979)

Submitted for Publication/In Press

G. N. Ling, M. M. Ochsenfeld, C. Walton and T. J. Bersinger - "Mechanism of Solute Exclusion from Cells: The Role of Protein Water Interaction(submitted to Science)

G. N. Ling - "The Theory of the Allosteric Control of Cooperative Adsorption and Conformation Changes: A Molecular Model for Physiological Activities According to the Association-Induction Hypothesis" in Cooperative Phenomena in Biology Ed. G. Karreman, Pergamon Press, Inc. (In Press)

G. N. Ling and W. Negendank - "Do Isolated Membranes and Purified Vesicles Pump Sodium - A Critical Review and Reinterpretation", Perspectives in Biology and Medicine, (In Press)

G. N. Ling and M. T. Tucker, "Nuclear Magnetic Resonance Relaxation and Water Contents in Normal Tissues and Five Types of Cancer Cells, Journal of National Cancer Institute (submitted for publication)

G. N. Ling and A. Fisher - "Cooperative Interaction Among Cell Surface Sites: Further Evidence in Support of the Surface Adsorption Theory of Cellular Electrical Potential, J. Cell. Physiol. (submitted for publication)

G. N. Ling, C. L. Walton, and T. J. Bersinger, "Reduced Solubility of Polymer-Oriented Water for Sodium Salts, Sugars, Amino Acids and Other Solutes Normally Maintained at Low Levels in Living Cells, Physiol. Chem. Phys.(submitted for publication)

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Donnan, F. G., The Theory of Membrane Equilibrium, Chem. Rev., 1:73-90 (1924)

Ruzyllo, W. and R. L. Vick, Cellular Resting and Diastolic Potentials in Canine Purkinje Cells: Effects of External $[K^+]$ and Repetitive Excitation, J. Mol. Cell. Cardiol., 6:27-37 (1974)

Weidemann, S., Elektrophysiologie der Herzmuskelfaser, Bern: Huber (1956)

$$\frac{(a^{i}_{H^+}/a^{o}_{H^+})^{Zi}}{(C_i)_i/C_i)_o}$$

Series:	I (n=4)	II (n=4)	III (n=4)	IV (n=4)	Total Average (n=8)
Na^{22}	0.55±0.05	0.54±0.08	0.56±0.04	0.53±0.09	0.54±0.06
K^{42}	-	-	-	-	0.25±0.03
Rb^{86}	0.71±0.08	0.50±0.04	0.58±0.04	0.59±0.13	0.58±0.07
Cs^{134}	0.50±0.07	0.59±0.11	0.47±0.09	0.61±0.09	0.54±0.06
Ca^{45}	2.14±0.47	2.42±0.68	2.82±0.64	1.75±0.30	2.28±0.39
Co^{60}	2.46±0.35	1.91±0.63	2.31±0.73	2.06±0.15	2.19±0.35
Zn^{65}	1.93±0.43	1.96±0.46	2.60±0.19	1.29±0.28	1.94±0.29
Cd^{109}	2.82±0.23	2.82±0.86	3.57±0.42	2.08±0.49	2.82±0.41
Fe^{59}	18.70±1.40	19.08±1.71	18.23±1.59	18.53±1.76	18.38±1.10

Legend

Series I : Samples where the isotope is added to the inside
Series II : Samples where the isotope is added to the outside
Series III: Samples having average incubation time of 49 days
Series IV : Samples having average incubation time of 97 days
n : Number of samples in a series

Table 1

Expt. No.	Ψ (mV.)	$a^i_{H^+}$ (mM)	$a^o_{H^+}$ (mM)	$\frac{a^i_{H^+}}{a^o_{H^+}}$	$a^i_{Cu^{++}}$ (mM)	$a^o_{Cu^{++}}$ (mM)	$\left(\frac{a^i_{Cu^{++}}}{a^o_{Cu^{++}}}\right)$ Observed	$\left(\frac{a^i_{Cu^{++}}}{a^o_{Cu^{++}}}\right)$ Theoretical (Donnan)	$\frac{\left(a^i_{H^+}/a^o_{H^+}\right)}{\left(a^i_{Cu^{++}}/a^o_{Cu^{++}}\right)}$ Observed
1	53.25	28.2	2.52	11.2	.080	0.012	6.66	125	1.68
2	59.23	25.2	2.19	11.8	0.074	0.006	12.33	139	0.95
3	45.78	21.9	3.89	5.62	0.065	0.030	2.17	31.6	2.59
4	51.65	23.5	2.51	9.33	0.087	0.012	7.25	87.0	1.29
5	18.38	13.5	6.61	2.04	0.035	0.048	0.73	4.16	2.79
6	59.79	25.2	2.52	10.0	0.080	0.013	6.15	100	1.63
7	50.56	22.4	2.69	8.32	0.074	0.014	5.29	69.2	1.57
8	54.75	24.0	2.95	8.13	0.090	0.017	5.29	66.1	1.54
9	60.74	24.6	2.03	11.8	0.080	0.005	16.0	139	0.73

Polystyrene sulfonic acid ca 2.2% (w/v)

TABLE

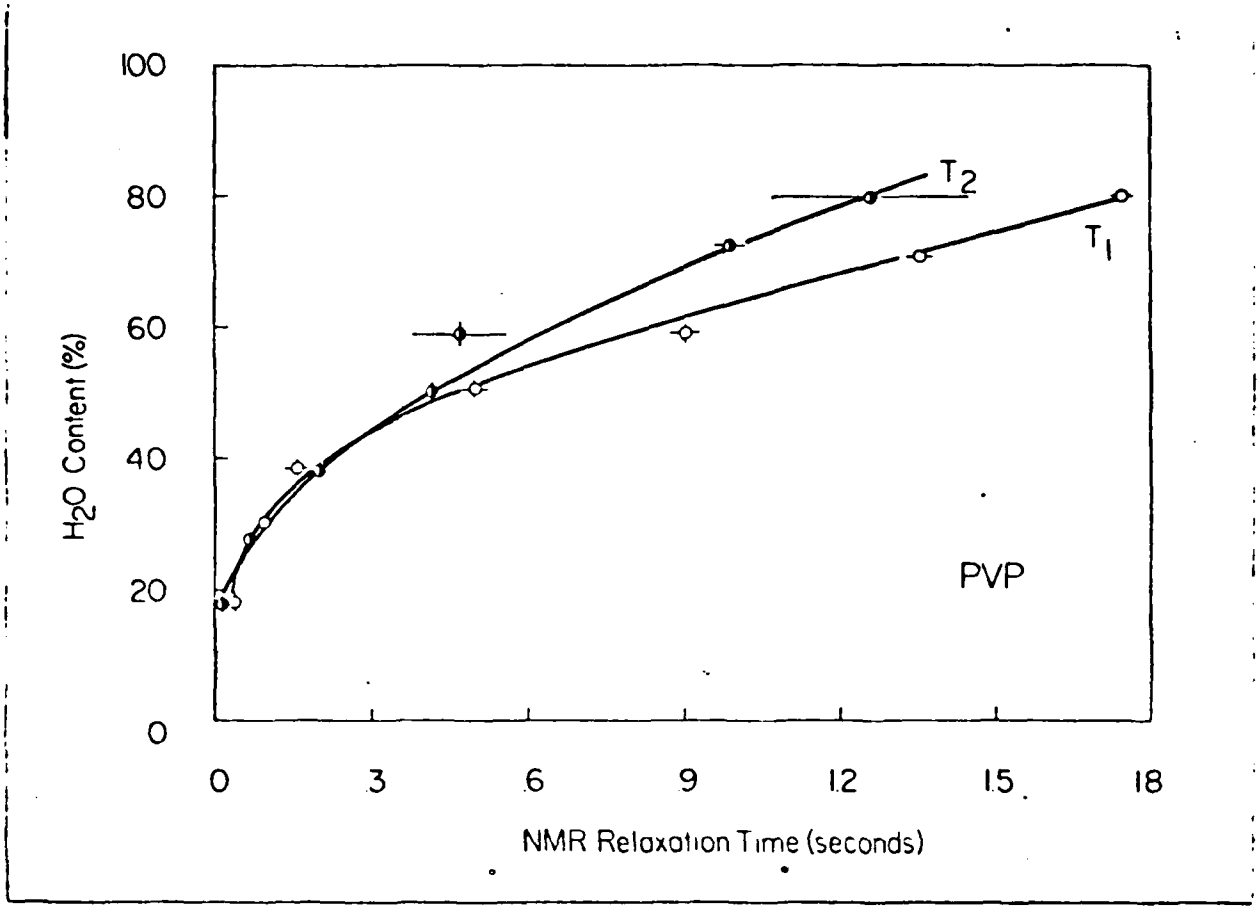


FIGURE 1

NMR Relaxation Times T_1 and T_2 of Water Protons in Polyvinylpyrrolidone-water Systems Containing Varying Percentages of Water T_1 Obtained with 180°-τ-90° Pulse Sequence T_2 with Carr-Purcell Spin Echo Method.

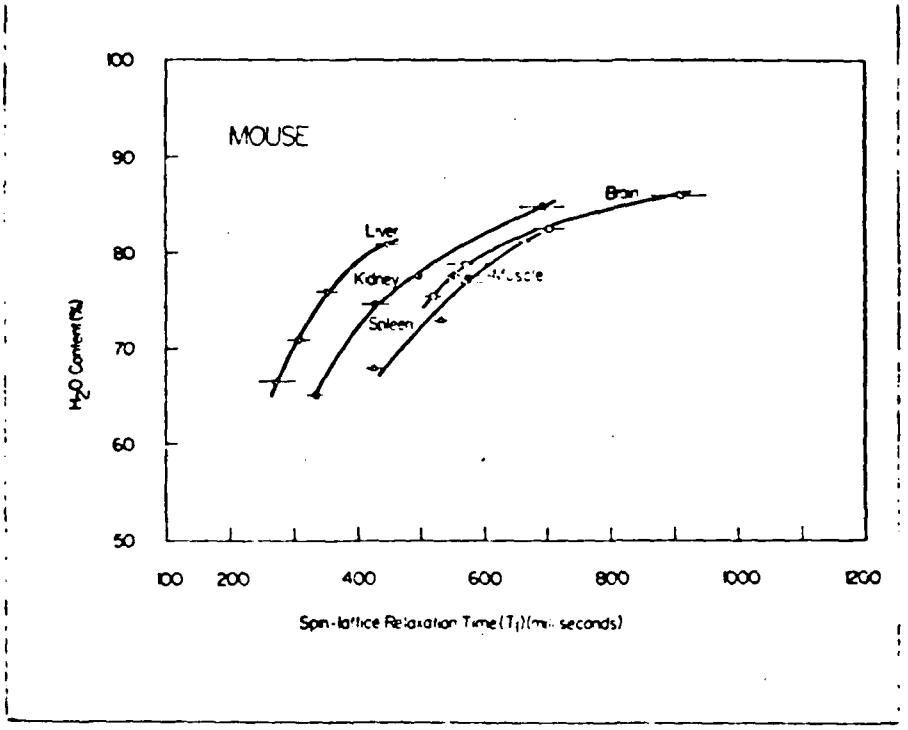
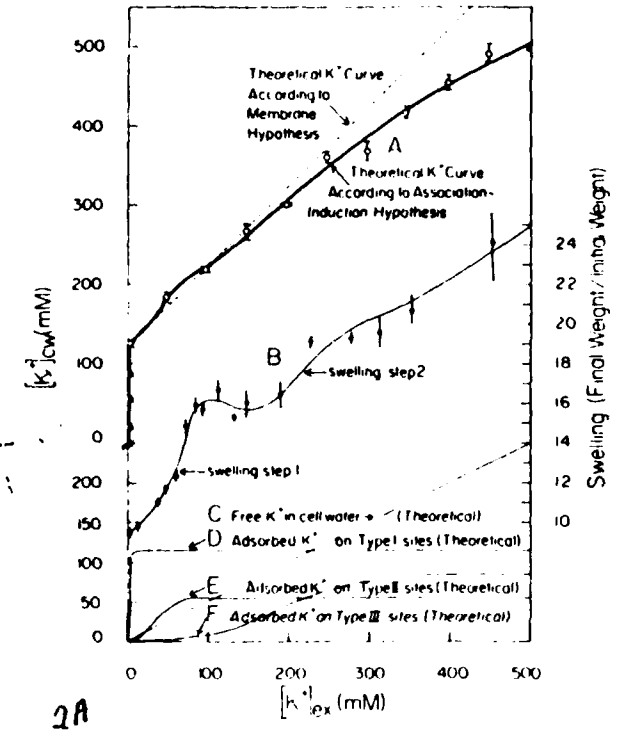


FIGURE 2

Spin-lattice Relaxation Time (T_1) vs. H_2O Content Plots of Several Normal Mouse Tissues. Water Contents were Varied by 2-hour Incubation in Hypotonic and Hypertonic (with added sucrose) Ringer Solution.



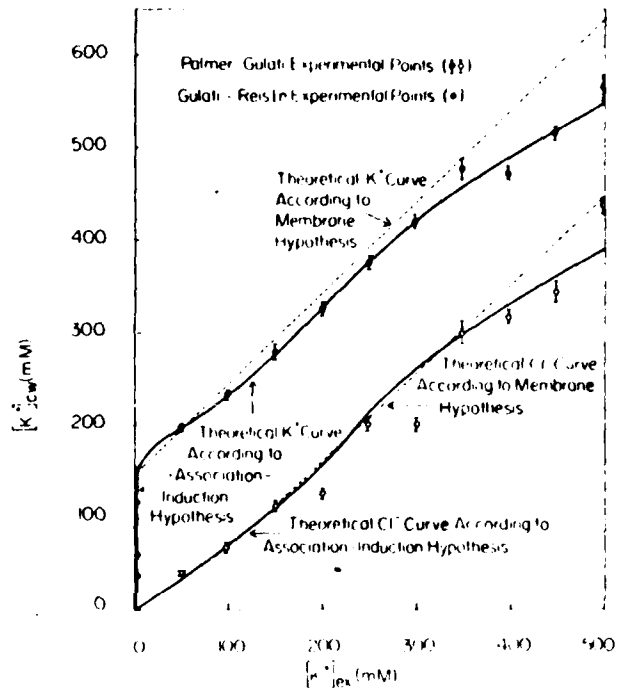


FIGURE 2A

Fig. 4 (left). Potassium concentration in frog muscle cells in the presence of 91 mM external NaCl. (○) New data on K^+ accumulation confirming those of Palmer and Gulati (*1*); (●) new data on muscle swelling, and (◐) old data of Ling and Bohr (*8*) on K^+ accumulation. Curve A is a theoretical curve derived from the explicit form of Eq. 1 (*3*), which is resolvable into components shown as curve C [free K(Cl)], curve D (type I adsorption), curve E (type II adsorption), and curve F (type III adsorption). The contribution of type I sites was determined from the results of previous studies (*7-9*); those of type II and type III sites were estimated from curve B, which records the two-step swelling of frog muscles under conditions similar to those of curve A, except that a low external NaCl concentration of 30 mM was used (*19*). The q value used to obtain curve C was 0.5. Other numerical values used to obtain curves D, E, and F, respectively, were $[F]_i$ = 122, 55, and 85 mM; K_i = 1.35, 35, and 185 mM; and $-\gamma/2$ = 0.54, 1.36, and 0.91 kcal/mole. For all data points the lengths of the error bars represent twice the standard error based on four or more determinations. The dashed straight line, predicted on the basis of the membrane theory as given by Palmer and Gulati (*1*), intercepts the ordinate at about 150 mM. Fig. 2 (right). Potassium and chloride in frog muscle cells. The experimental points are from Palmer and Gulati (*1*) and Gulati and Reisin (*10*) as indicated. Solid curves were derived from the explicit form of Eq. 1 (*3*). Dashed lines were derived on the basis of the membrane theory. The numerical values used to obtain the theoretical curves for K^+ were q = 0.5 for curve C and, for curves D, E, and F, respectively, $[F]_i$ = 150, 12, and 120 mM; K_i = 1.0, 28, and 210 mM; and $-\gamma/2$ = 0.60, 1.36, and 0.91 kcal/mole. The theoretical curve of Cl accumulation is equal to that for K^+ accumulation minus type I adsorption.

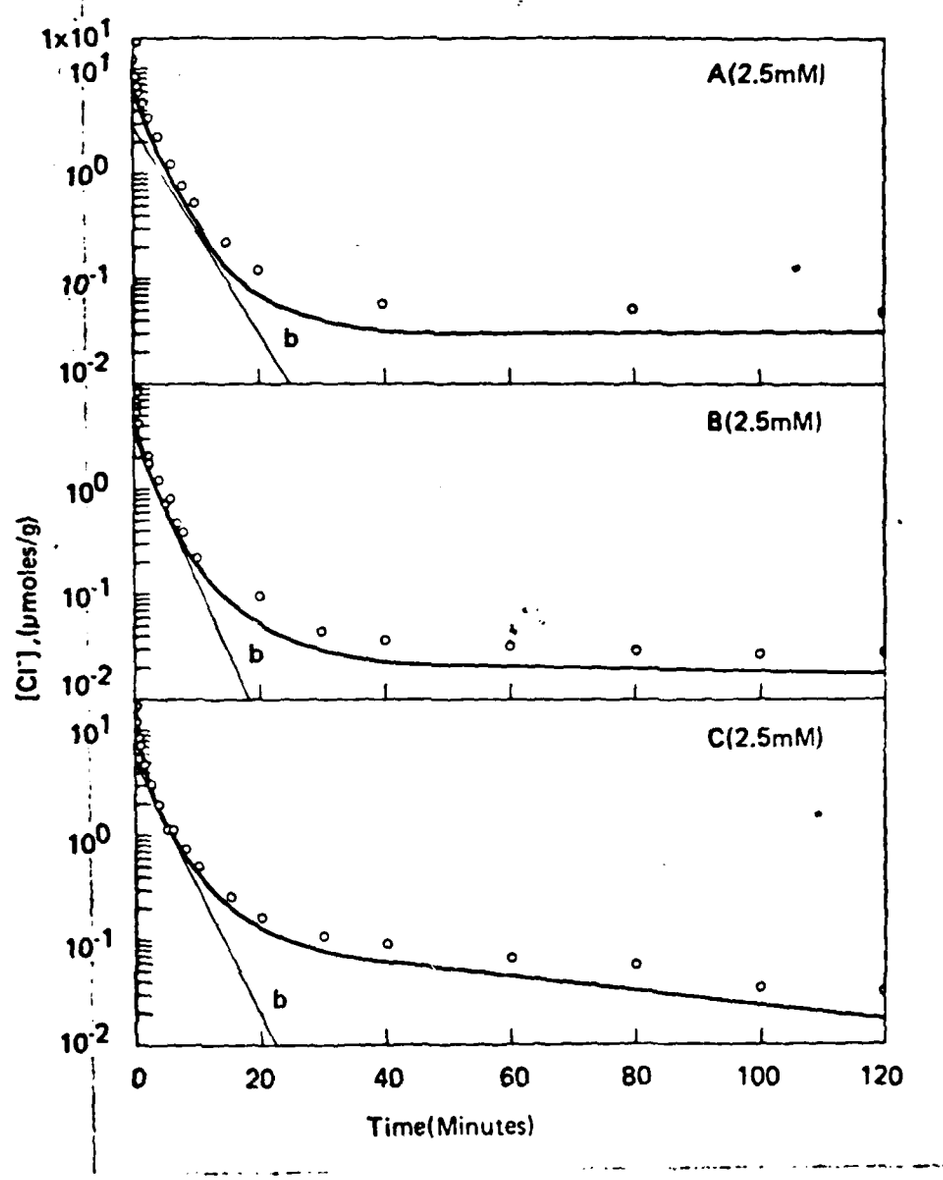

A(2.5mM)
B(2.5mM)
C(2.5mM)
b
[Cl⁻], (μmoles/g)
1x10¹
10¹
10⁰
10⁻¹
10⁻²
0
20
40
60
80
100
120
Time(Minutes)

FIGURE 3

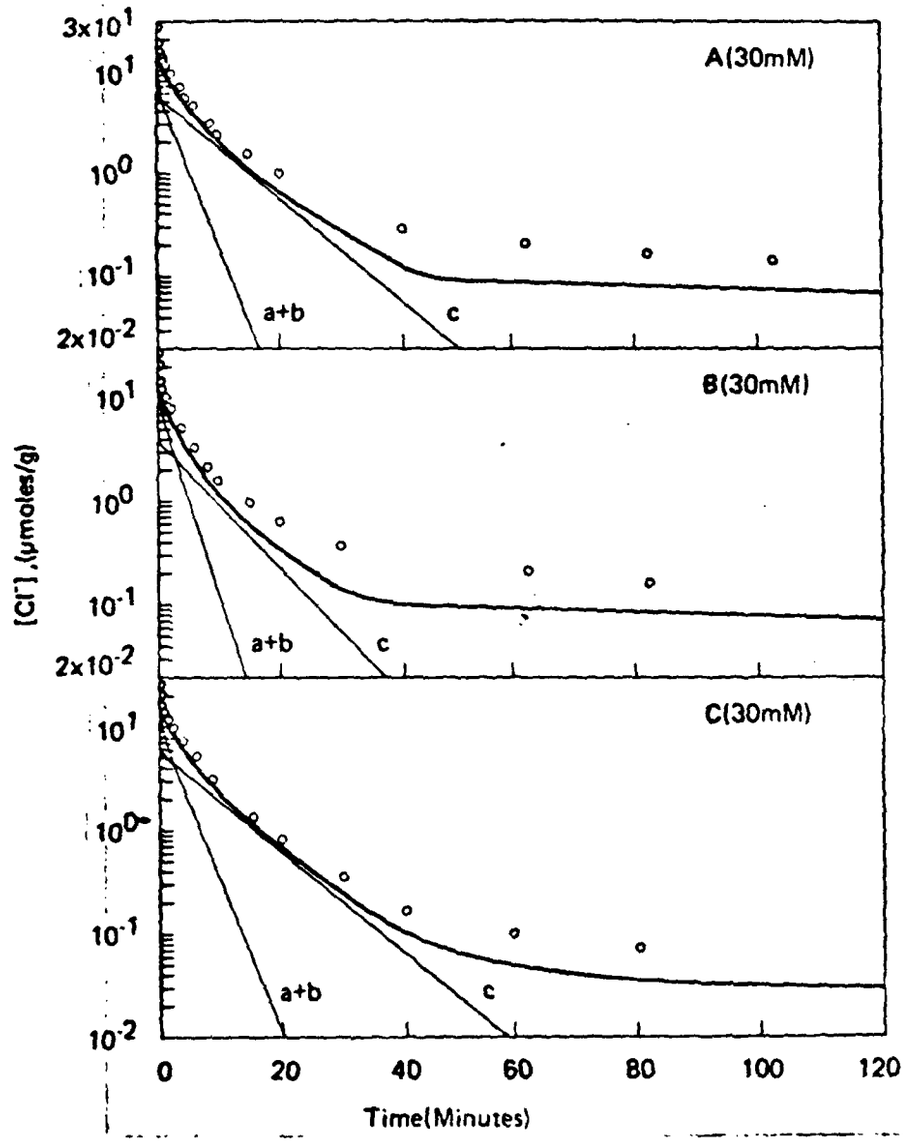

A(30mM)
B(30mM)
C(30mM)
a+b
c
[Cl⁻], (μmoles/g)
Time(Minutes)
3x10¹
10¹
10⁰
10⁻¹
2x10⁻²
10⁻²
0
20
40
60
80
100
120

FIGURE 4

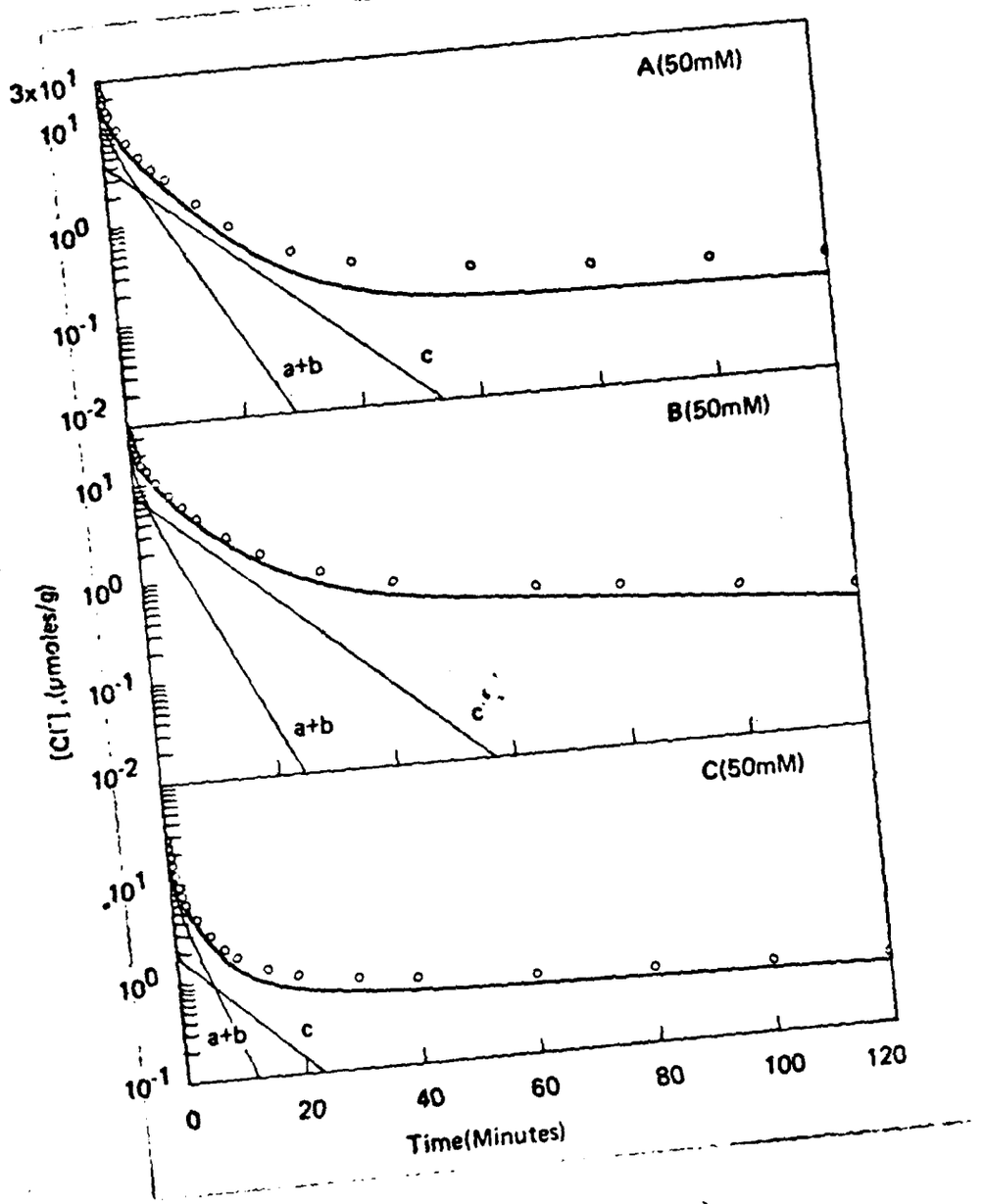

A(50mM)
B(50mM)
C(50mM)
a+b
c
3x10¹
10¹
10⁰
10⁻¹
10⁻²
[Cl⁻] (μmoles/g)
0
20
40
60
80
100
120
Time(Minutes)

FIGURE 5

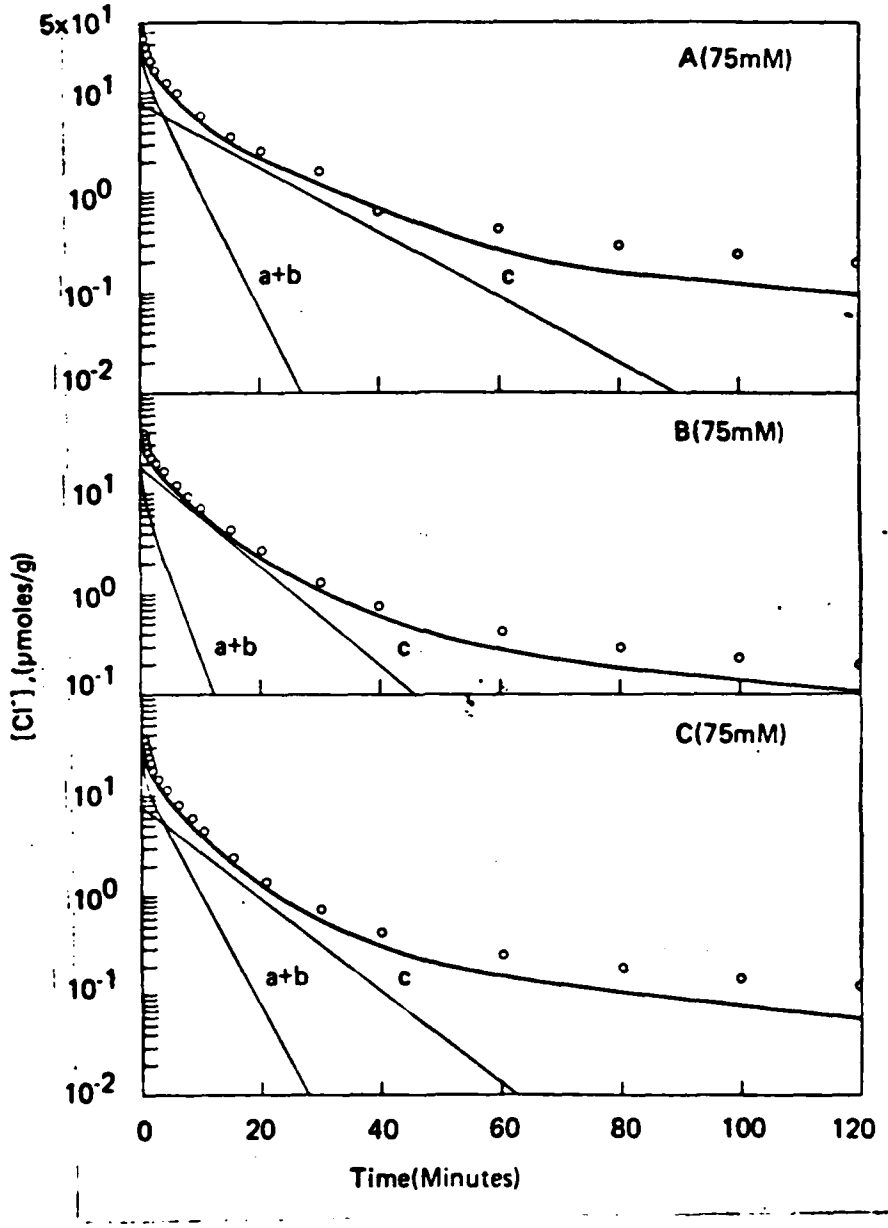

5x10¹
10¹
10⁰
10⁻¹
10⁻²
A(75mM)
a+b
c
B(75mM)
a+b
c
C(75mM)
a+b
c
[Cl⁻], (μmoles/g)
0
20
40
60
80
100
120
Time(Minutes)

FIGURE 6

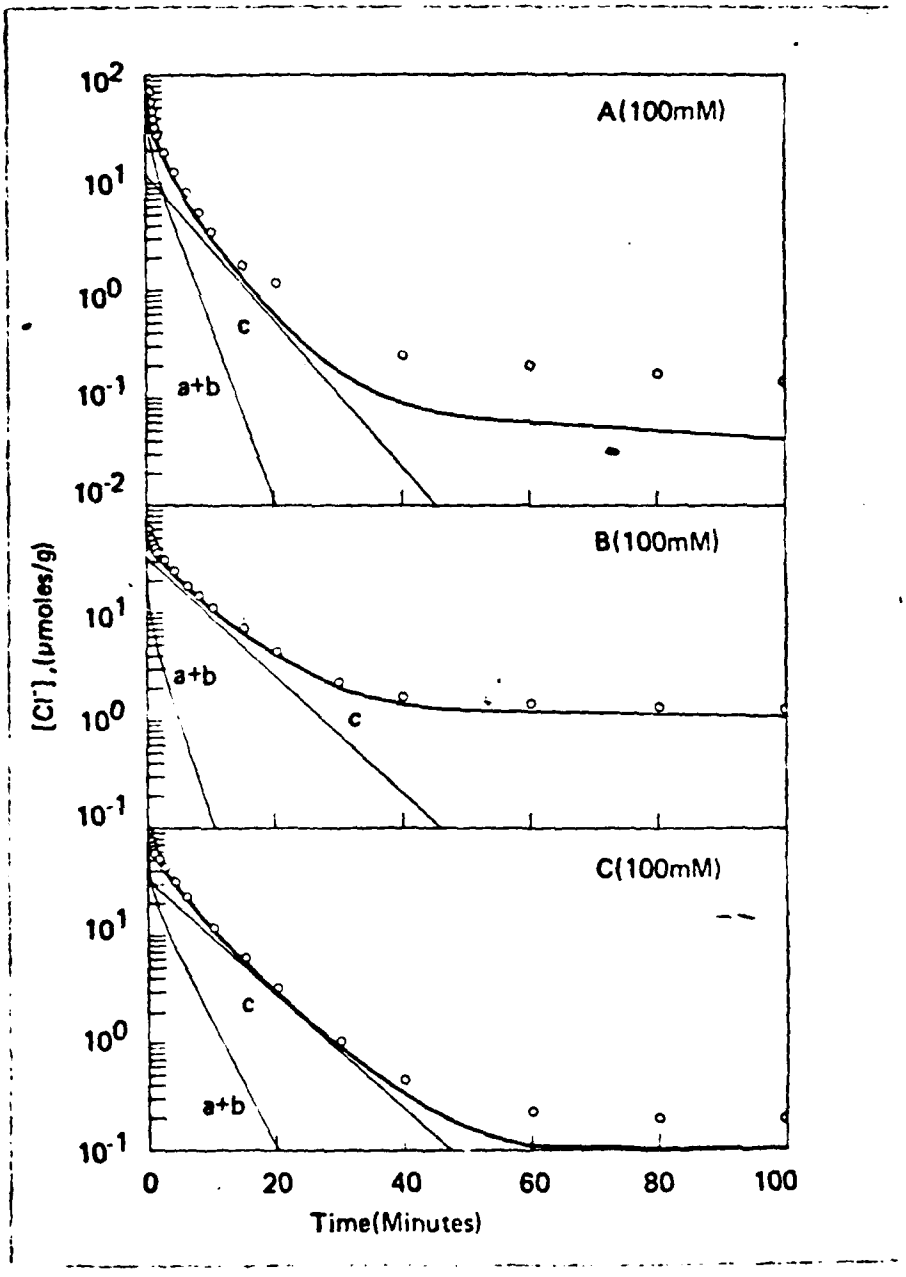

A(100mM)
B(100mM)
C(100mM)
a+b
c
[Cl⁻], (μmoles/g)
Time(Minutes)
10²
10¹
10⁰
10⁻¹
10⁻²
0
20
40
60
80
100

FIGURE 7

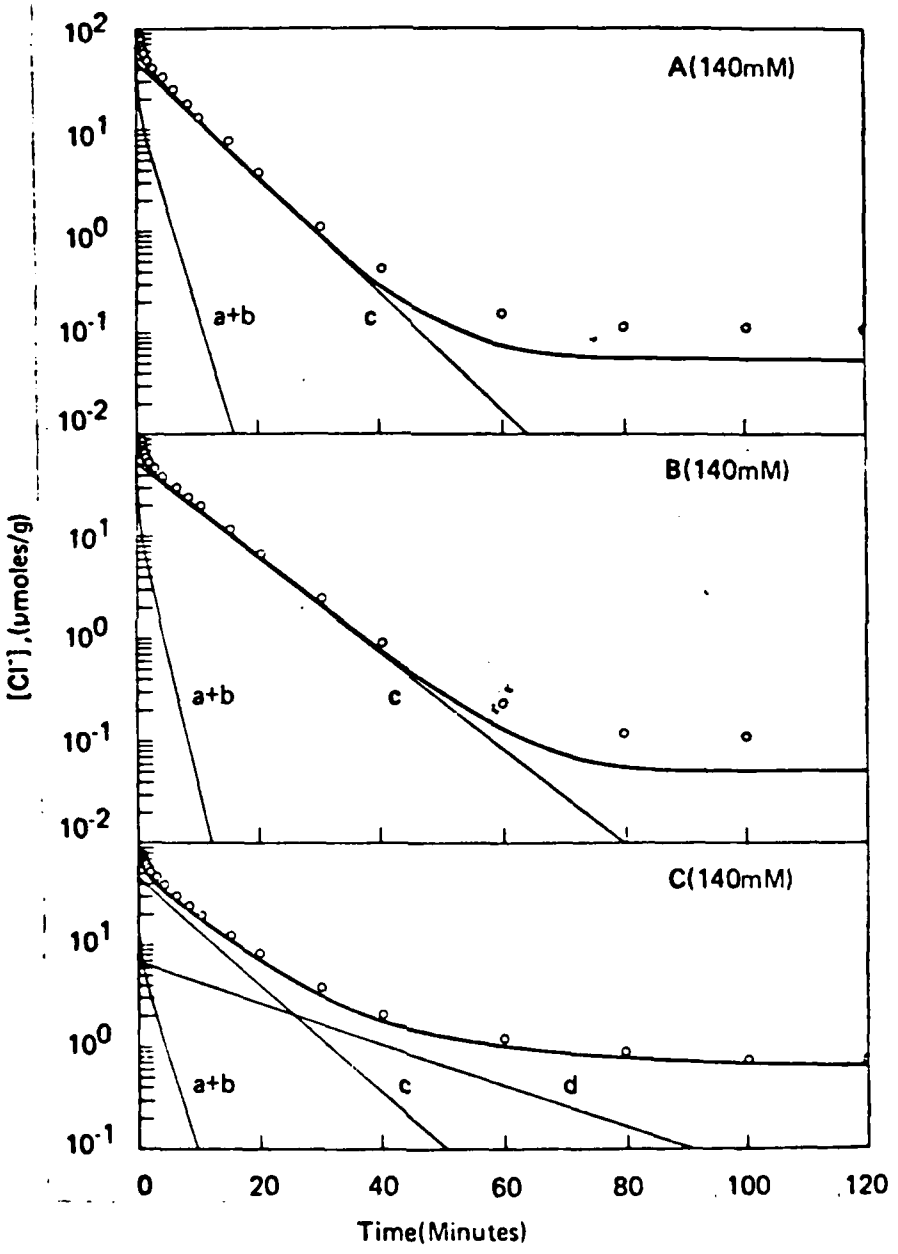

A(140mM)
B(140mM)
C(140mM)
a+b
c
d
tot
[Cl⁻], (μmoles/g)
Time(Minutes)
10²
10¹
10⁰
10⁻¹
10⁻²
0
20
40
60
80
100
120

FIGURE 8

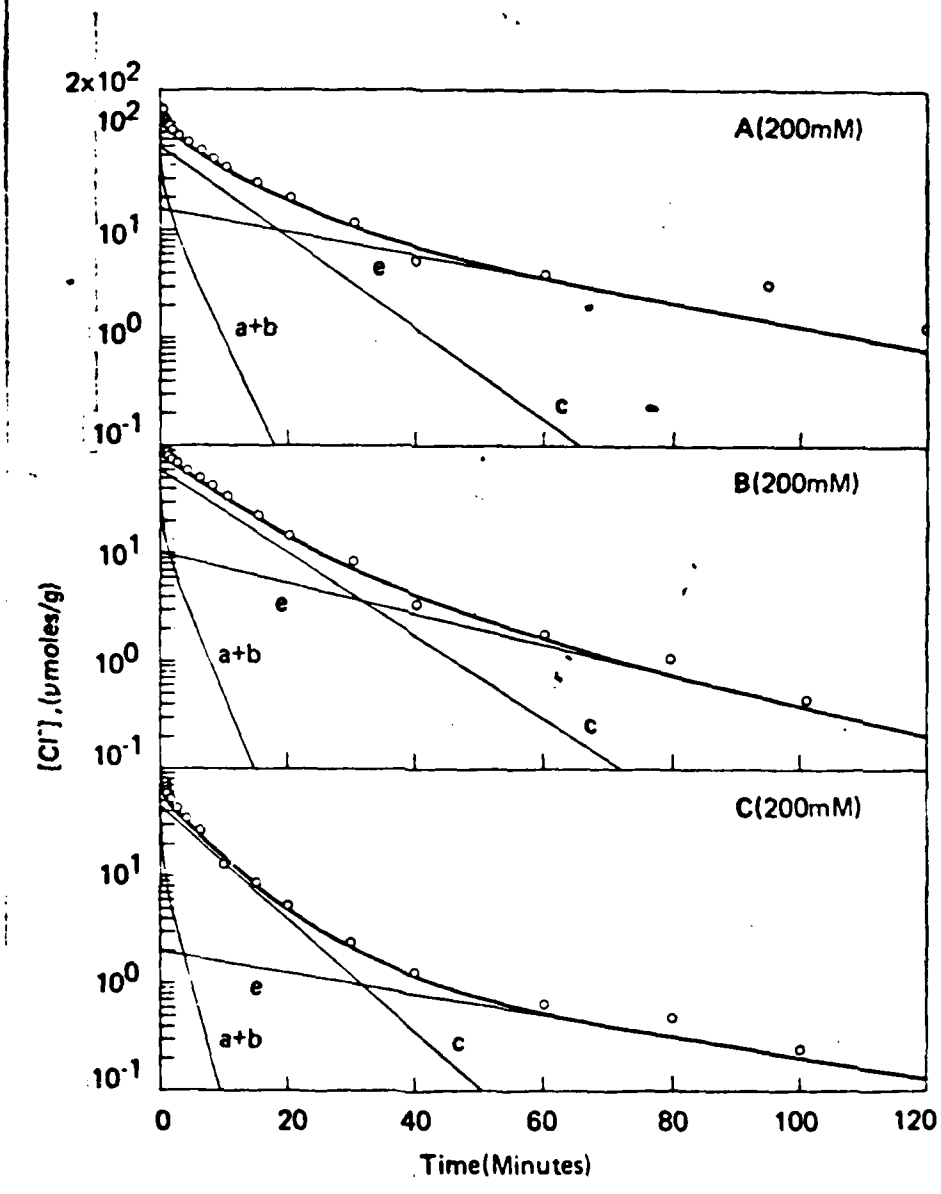

2x10^2
10^2
10^1
10^0
10^-1
A(200mM)
B(200mM)
C(200mM)
e
a+b
c
[Cl⁻], (umoles/g)
0
20
40
60
80
100
120
Time(Minutes)

FIGURE 9

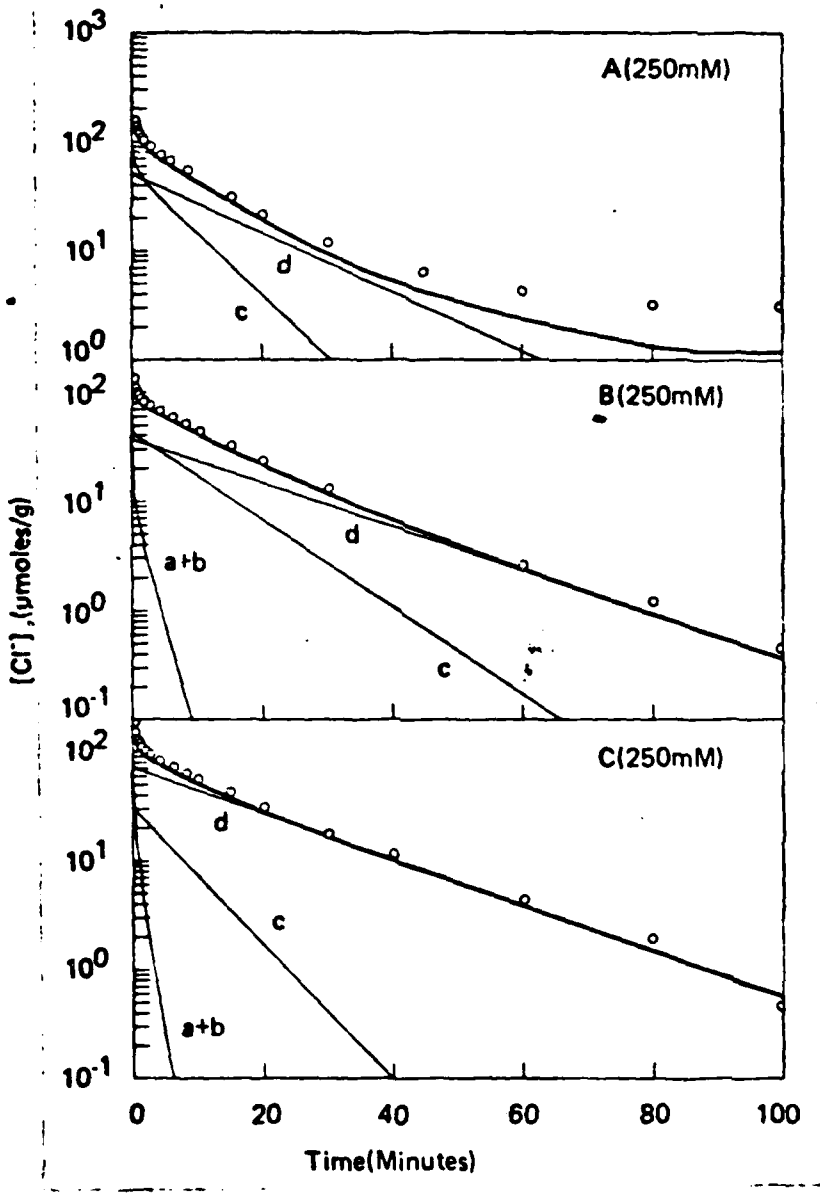

A(250mM)
B(250mM)
C(250mM)
d
c
a+b
[Cl⁻], (μmoles/g)
10³
10²
10¹
10⁰
10⁻¹
0
20
40
60
80
100
Time(Minutes)

FIGURE 10

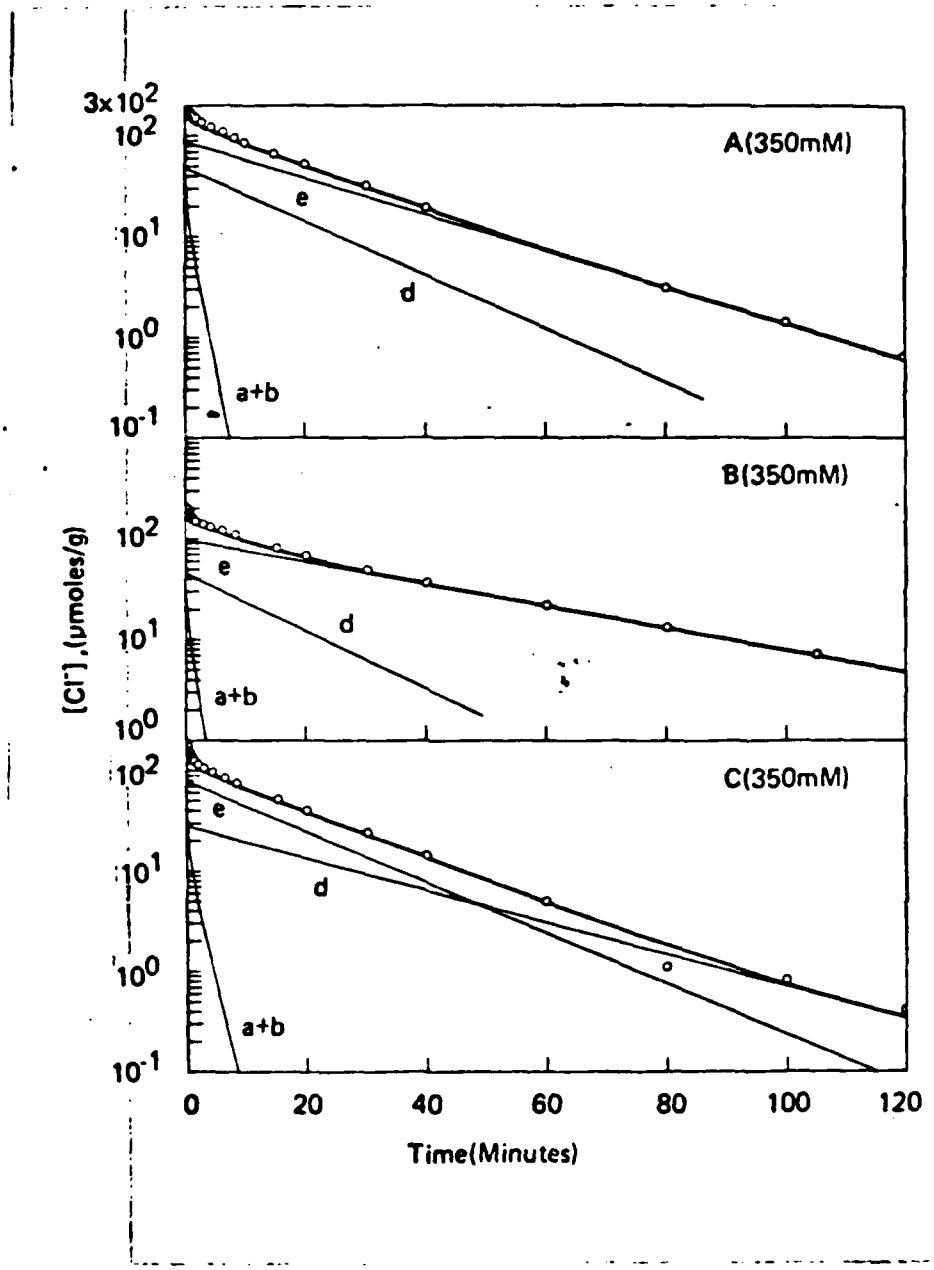

3x10^2
10^2
10^1
10^0
10^-1
A(350mM)
B(350mM)
C(350mM)
e
d
a+b
[Cl⁻], (μmoles/g)
0
20
40
60
80
100
120
Time(Minutes)

FIGURE 11

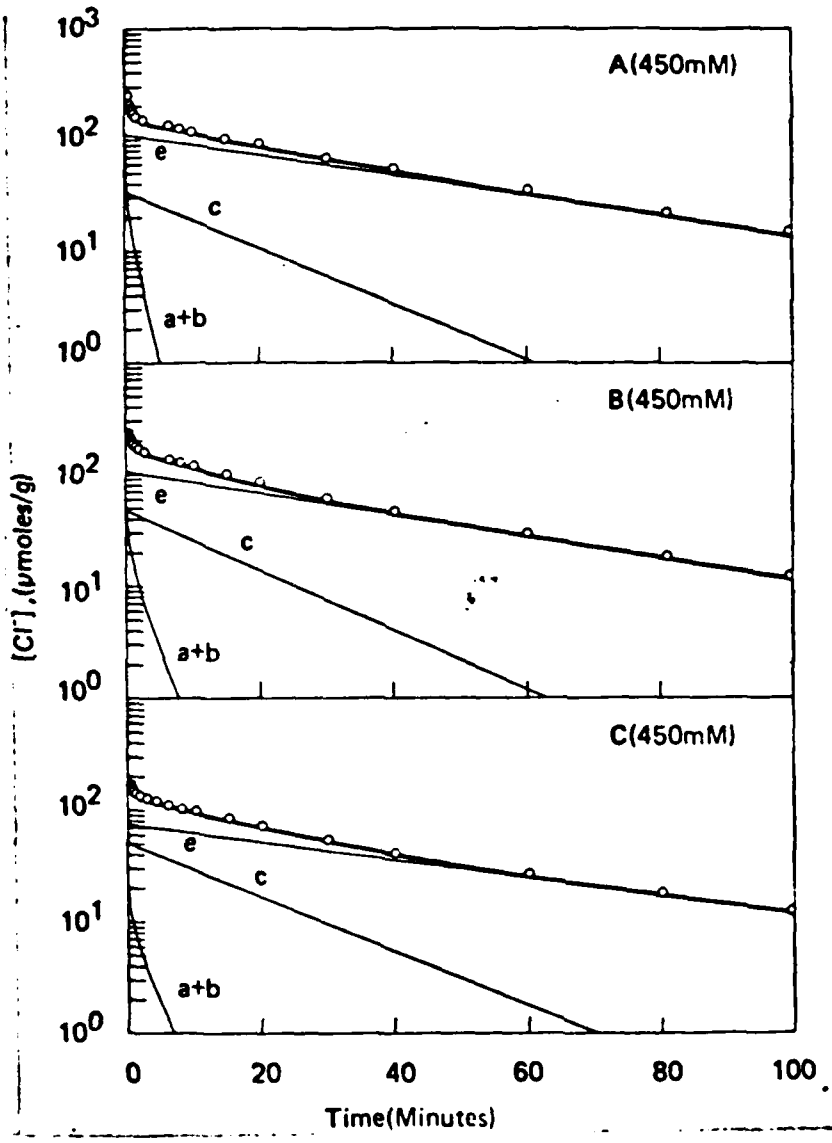

A(450mM)
B(450mM)
C(450mM)
e
c
a+b
10^3
10^2
10^1
10^0
[Cl⁻], (μmoles/g)
0
20
40
60
80
100
Time(Minutes)

FIGURE 12

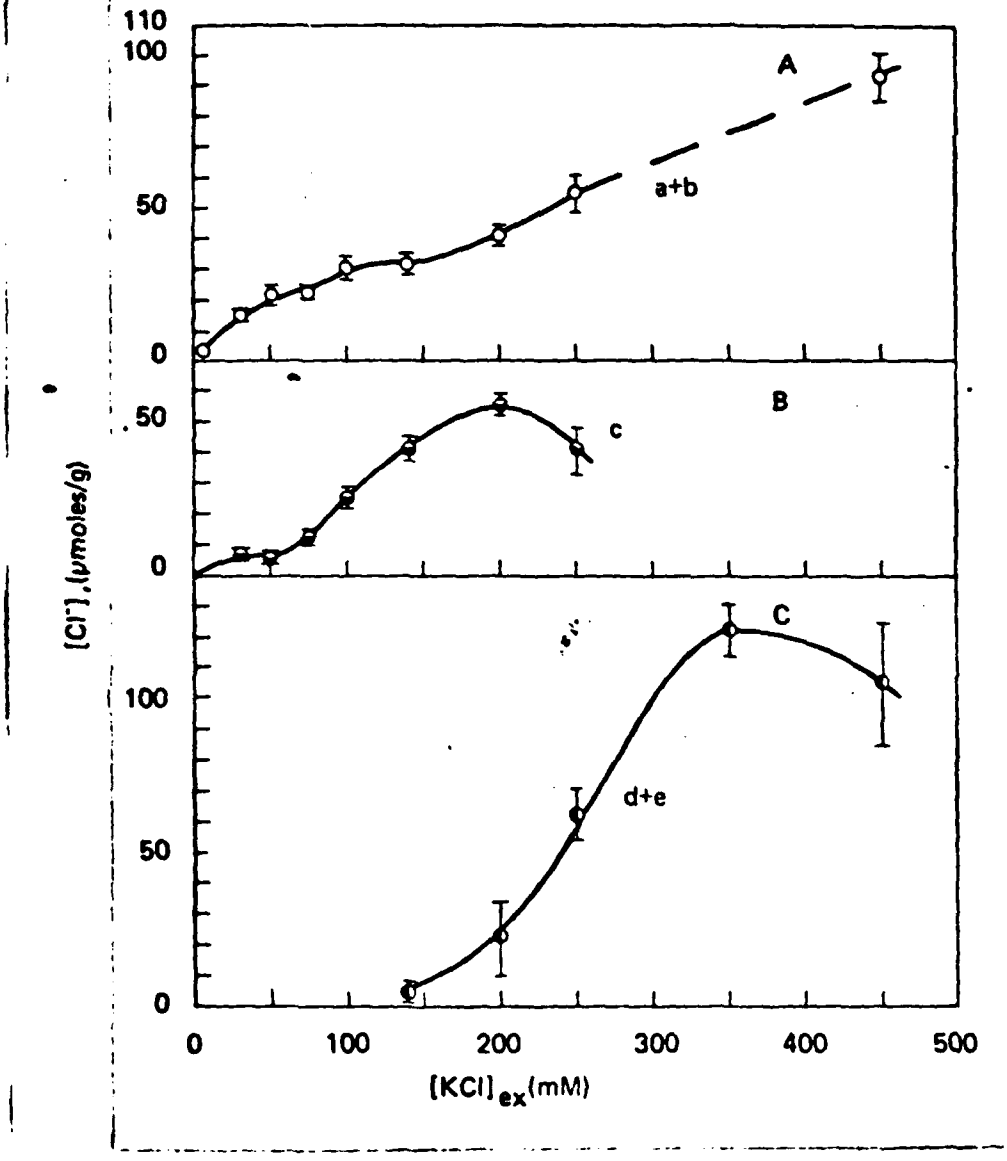

110
100
50
0
A
a+b
50
0
B
c
100
50
0
C
d+e
0
100
200
300
400
500
[Cl⁻], (μmoles/g)
[KCl]ex(mM)

FIGURE 13

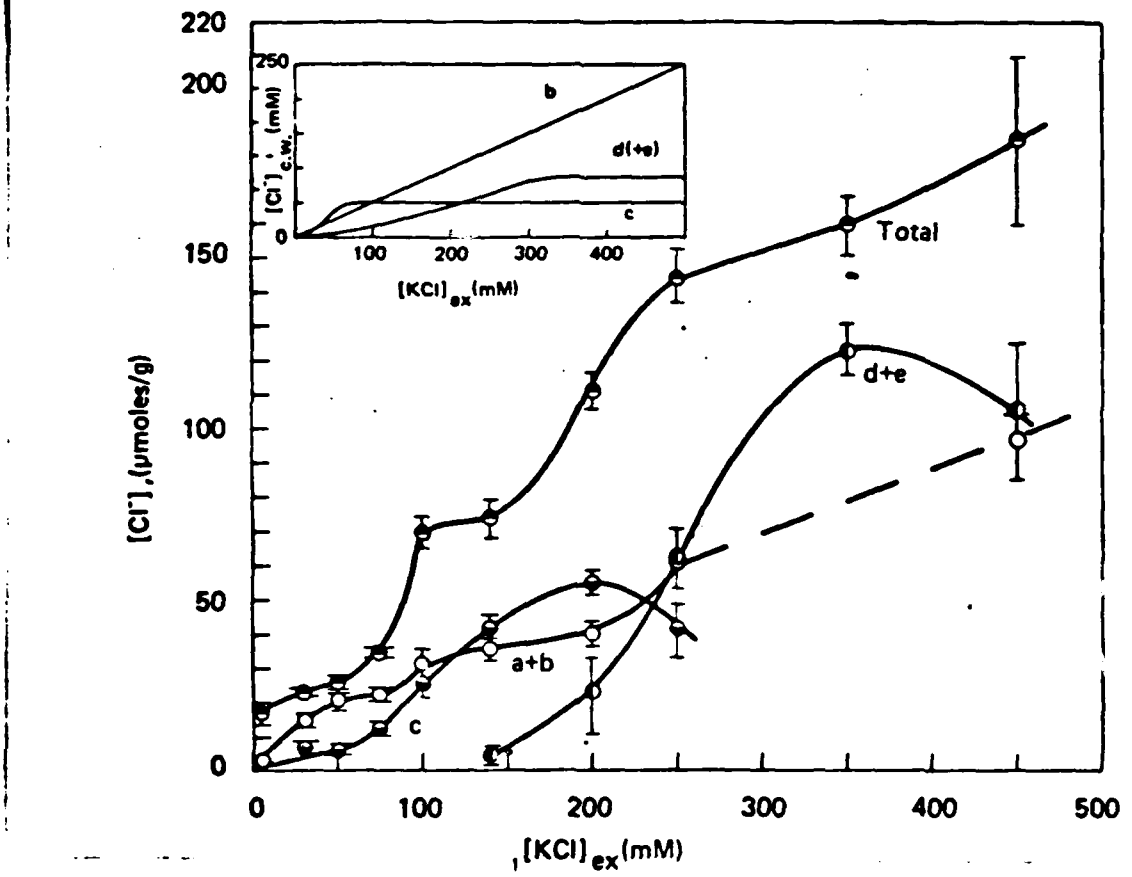

220
200
150
100
50
0
[Cl⁻], (μmoles/g)
0
100
200
300
400
500
[KCl]ex (mM)
Total
d+e
a+b
c
250
200
150
100
50
0
[Cl⁻]c.w. (mM)
b
d(+e)
c
100
200
300
400
[KCl]ex (mM)

FIGURE 14

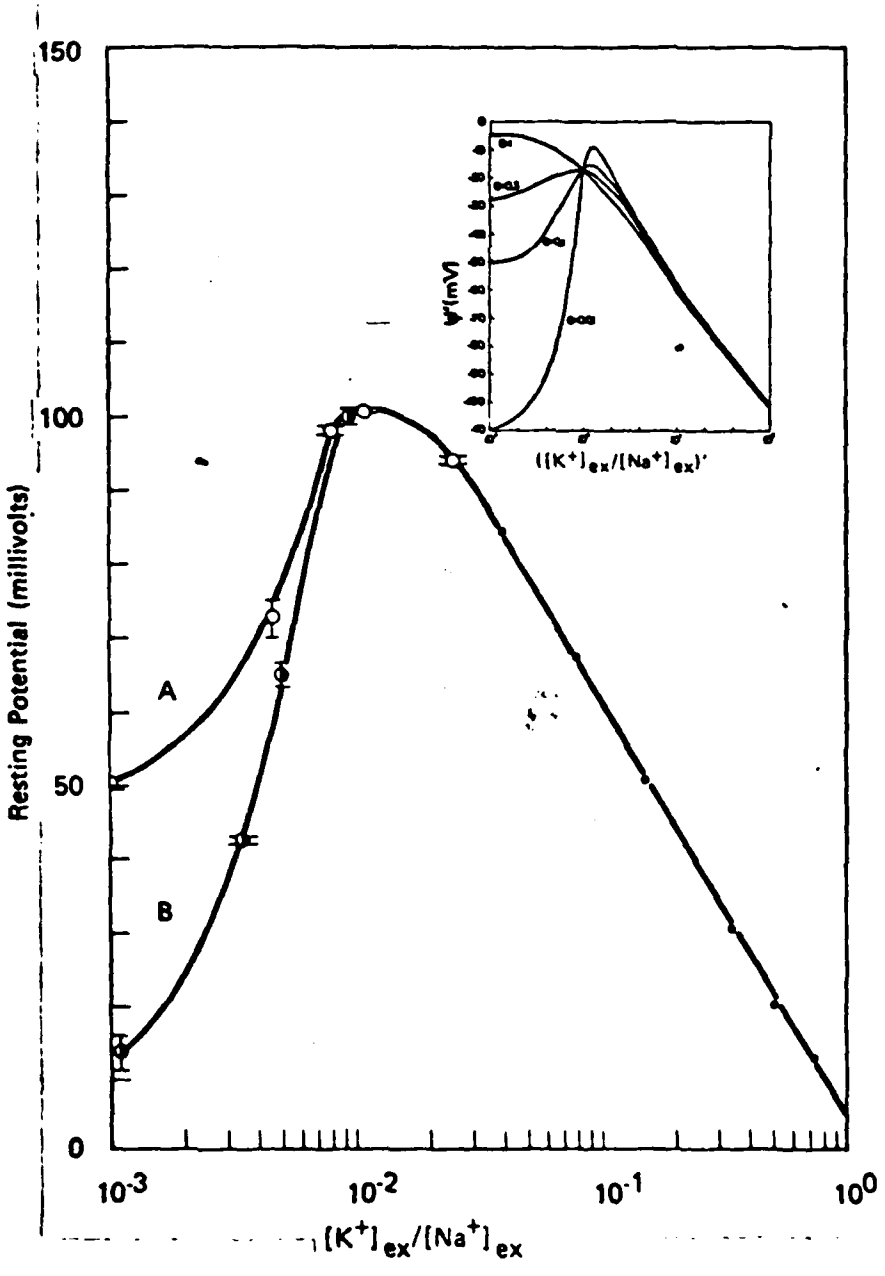
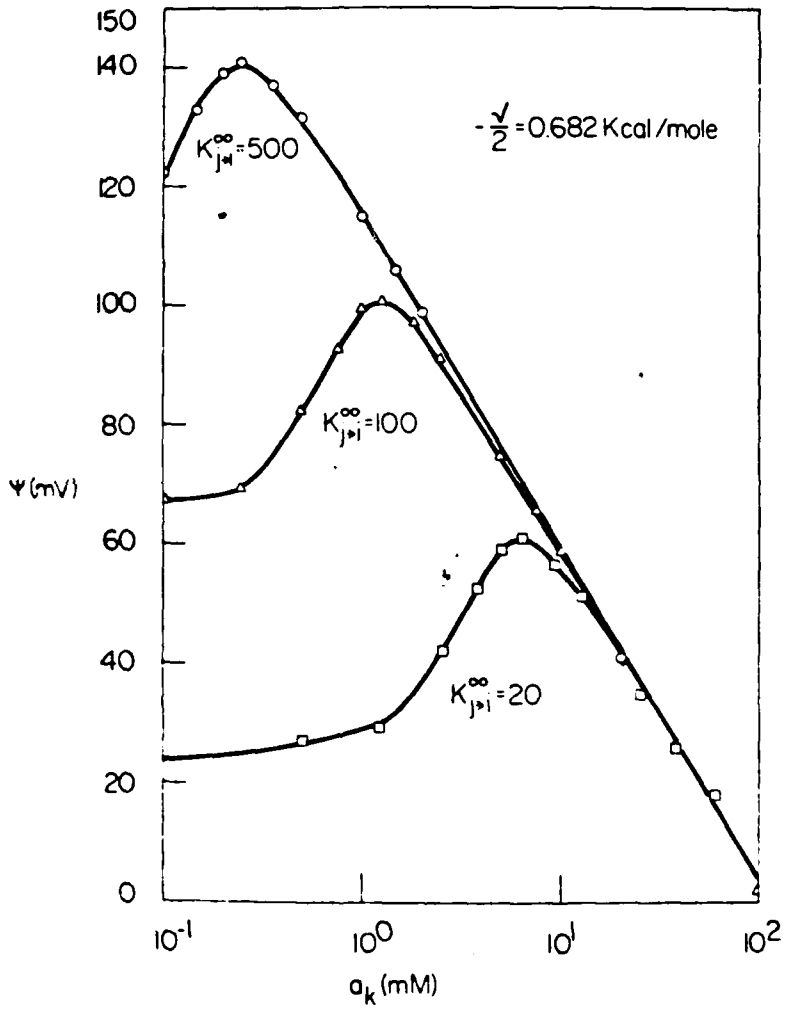
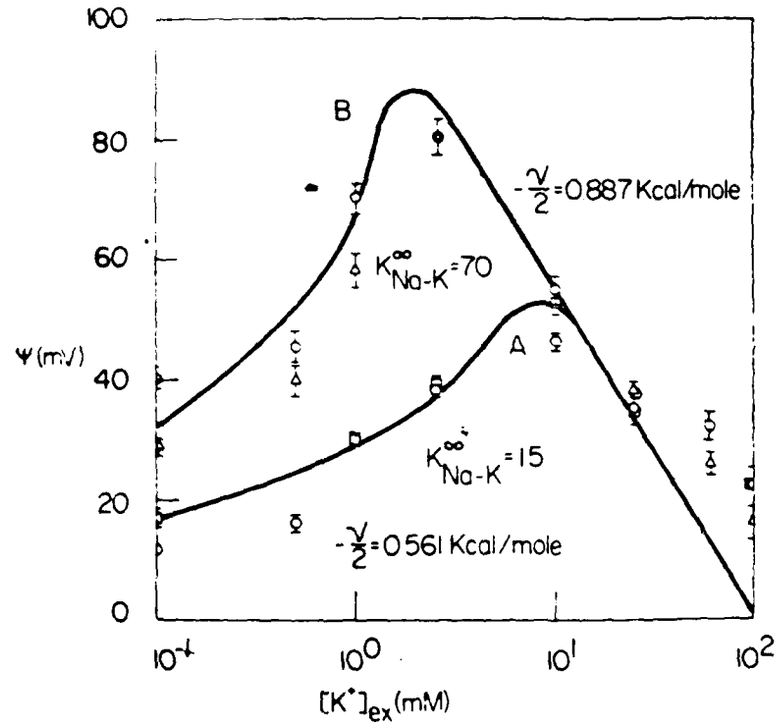

150
100
50
0
Resting Potential (millivolts)
A
B
10^{-3}
10^{-2}
10^{-1}
10^{0}
$[K^+]_{ex}/[Na^+]_{ex}$
$([K^+]_{ex}/[Na^+]_{ex})'$

FIGURE 15



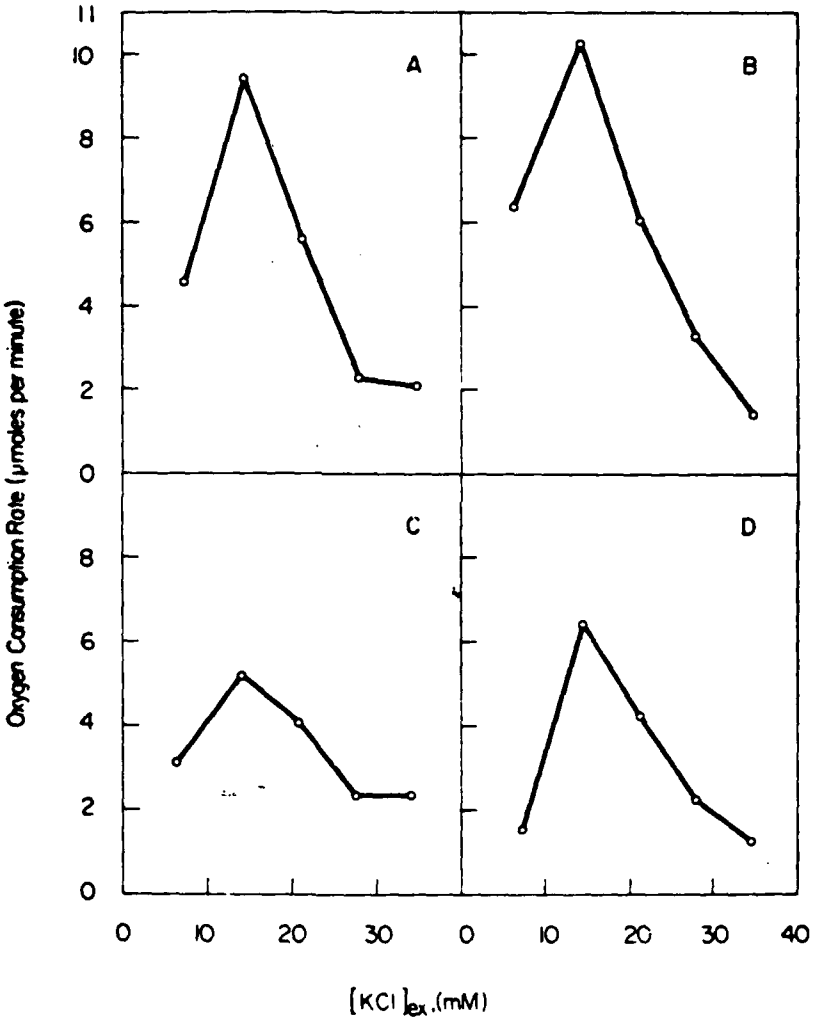
[illegible] curves of the resting potential according to [illegible]. Curves are calculated for $-\gamma/2 = 0.682$ [illegible] but varying $K^{\infty}_{j\to i}$: 500, 100 and 20.

FIGURE A



...: Curve A showed resting potential at varying external K^+ concen-
... at ... X 10^{-7} M. Equilibrium was allowed after 2 hours of
... B was recorded 4 hours later after ... Curve B
... resting potential has returned completely to normal. Solid lines
... resting potential corresponding to $-\nu/2$ and K^{∞}_{Na-K} value as shown. Data
... effect of ouabain on the resting potential ... surface sites.

FIGURE 8



Effect of external KCl concentration on rate of oxygen consumption of isolated frog sartorius muscles. The oxygen consumption rate refers to the basis of two sartorius muscles, other weights were in the range of 160 to 250 mg and not given in the graph.

FIGURE C

Progress Report

For Part of the Work Accomplished Between
Sept 1, 1979 - to Aug 31, 1980
under ONR Contract N00014-79-C-0126

Principal Investigator:

Gilbert N. Ling, Ph.D.
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1. Disproof of the Lipid Membrane Theory of Overton

The membrane-pump theory is built on Overton's lipoidal membrane theory. In its current version, lipid layer *not only* furnishes the semipermeable barrier of the cell; it also provides the enclosing water-immisible fluid phase to harbour the postulated "carriers" and "pumps". Obviously to serve these roles the lipid layer must cover the whole cell surface. Indeed the electron microscopic demonstration of the presence of a tri-layered "unit membrane" around many types of living cells led at one time to the belief that this is indeed the case and that the lipid membrane in general and the "Paricimolecular" model of the cell membranes in particular was confirmed. Subsequent studies made it difficult, if not impossible, to sustain this belief. Thus it was found that extraction of 95% of the lipids did not alter the thickness nor the spacing of the laminar structure of the unit membrane (Fleischer et al, 1967; Morowitz and Terry, 1969). On the other hand, trilaminar structure was also demonstrated at the surfaces of "microspheres" prepared from lipid-free, pure proteinaceous materials (Fox et al, 1969).

Other major developments in the field of membrane research included the perfection of the technique of preparing phospholipid bilayers by Mueller and Rudin (1969). Thus a phospholipid bilayer by itself has extremely low ionic conductance when it separates two bodies of isotonic KCl solutions. Introduction of the K^+ specific ionophore, valinomycin (10^{-7} M) or monactin (10^{-6} M) increased the K^+ permeability of the lipid bilayer by several orders of magnitude (for review, see Jain, 1972). Thus willy-nilly these investigators had provided a powerful tool to test one of the most basic assumptions of membrane pump theory; the assumption that phospholipid layer provides the permeability barrier of plasma membranes and membranes of subcellular particles. The result of investigations using this tool was dramatic.

Valinomycin which increased K^+ permeability of man-made phospholipid layer drastically (Fig. 1) had no effect whatsoever on the conductance of mouse mitochondrial inner membrane (Moloff et al, 1978) nor does monactin have any detectable effect on K^+ permeability of giant squid axons (Stillman et al, 1970). Neither valinomycin, nor monactin nor nonactin had any effect on the K^+ permeability of the plasma membranes of frog muscle and ovarian eggs (Ling and Ochsenfeld, see Fig. 2).

These findings demonstrated conclusively that in most living cells and subcellular particles, the surface semi-permeable barrier is not that of a continuous lipoid layer - a fundamental disproof of Overton's lipoidal membrane theory, on which, alas, the entire membrane-pump theory was built.

2. Osmotic Activity of a (Non-charged) Polymer-water System

In recent years, three different laboratories across the world, but especially that of Ludwig Edelmann had presented unequivocal evidence that the bulk of K^+ in frog muscle cells is in an adsorbed state. This finding has added another major disproof of the membrane-pump theory. Since K^+ is the only major cation in the living cells, its adsorption leaves an osmotic deficit which cannot be made up within the confines of the basic tenets of the membrane theory (e.g., free K^+, free water).

In terms of the AI hypothesis, the deficit in the osmotic activity due to K^+ adsorption is neatly balanced by the direct effect of the extended matrix protein chains present in all living cells on the bulk phase cell water. That is to say, in living cells the osmotic activity (which is just an expression of the lowering of water activity) is brought about not by free ions but the extended protein chains.

That extended protein backbone acts on bulk-phase water has been clearly demonstrated in data presented in the attached reprint No. 5 . Furthermore we also clearly showed in this article that this water polarizing activity is shared with synthetic polymers such as PVP and poly(ethylene oxide) or PEO. Both are uncharged polymers, containing oxygen atoms at distances roughly two-water diameters apart.

We prepared a PEO solution of 30% (W/V). At a M.W. of 600,000, the total molarity of this polymer is only 300/600,000 = 0.5 mM. Thus in terms of total molarity, this 30% PEO solution should have no measured oxmotic activity.

In fact, as shown in Figure 3 the osmotic activity measured is higher than 1 M! These findings confirm the AI hypothesis concerning the basic mechanism of osmotic activity of living cells, i.e., due mainly to a matrix of proteins existing throughout the cell in an extended conformation.

3. The Physical State of Na^+ in Ion Exchange Resins

It was the fundamental assumption of the AI hypothesis since its very beginning 28 years ago that an ion exchange resin selectively accumulates K^+ (or Na^+) because it is that species of ion that is stoichomatically adsorbed onto the anionic sites. Although intuitively and theoretically reasonable, up to now, there has been no clear-cut direct experimental evidence for this assumption. Indeed, many seem to favor Gregor's theory of ion selectivity based on the opposite assumption - all counterions are free.

Quite aside from its basic role as the model of selective K^+ adsorption, Na^+ in ion exchange resins were also shown by Cope to share NMR characteristics with Na^+ in many living tissues. That is, the Na^+ in the resin and in living cells are not all NMR "visible" but only partly visible. It was based on the assumption that Cope and

Ling indirectly deduced that the bulk of K^+ in cells is adsorbed. (This conclusion has been extensively established by other means.)

Berendsen and Edze's attack on this original interpretation of NMR data on cell Na left a void - the question remained unanswered even though Chang and Woesner offered an excellent theoretical defense of the original binding interpretation. If the NMR signal undergoes a 40-60 split, does such a 40-60% split as was seen both in ion exchange resins and in living cells, constitute a sign that the Na^+ exists in a free state but under the influence of a diffuse electric gradient, or is in fact a close 1-on-1 adsorption?

To answer this question, we took advantage of the fact that ion exchange resin is only a cross-linked linear polystyrene sulfonate or polymethacrylate. Thus if one can prove that counterions are in fact adsorbed in a solution of these linear polymers, by deduction one can conclude first that Na^+ must be also adsorbed in ion exchange resins - and secondly living cells which share the 40-60 splitting of ^{23}Na NMR signals must also be in a state of 1-on-1 adsorption.

To test whether the counterion Na^+ is adsorbed or not, we prepared a solution of polystyrene sulfonate (TL-500) and polymethacrylate and dialyze off surplus Na^+. The total Na^+ in the solution was then measured by atomic absorption spectroscopy and the free Na activity measured with Na^+ ion specific electrode (Corning) the difference yielding the "bound" fraction.

Table 1 shows that in a 1% polymer solution of both TL-500 and PMA, roughly half of the counterion Na is measured by the electrode, the other half non-measurable.

However by itself such measurement only tells that half of the Na^+ cannot react with the electrode. It could indicate adsorption or simply occlusion in some secluded space between the polymer chains.

To prove that the remaining Na^+ is truly adsorbed one Na^+ on one anionic site we relied on the method of specific displacement of the adsorbed Na^+. That is to say, if the non-detected Na^+ is only freely floating in some inaccesible place, it should be displaceable by <u>any</u> monovalent cations. On the other hand, if it is specifically adsorbed, then differences in the short range attributes of the displacing ions will produce significantly different degree of displacement as revealed by an increase in measurable Na^+. Table 1 shows that indeed this is the case. Thus 59% of the Na in PMA was displaced and becomes detectable by arginine HCl, while an equal concentration of lysine HCl only displaced 15.9%. Similarly arginine HCl displaced 90.1% of Na adsorbed on TL-500 but only 54.5% in lysine.

We conclude that counterion in ion exchange resin and therefore in living cells exists primarily in a 1-on-1 adsorbed state.

4. Other Progress

a. I have critically reviewed the theory of oxidative phosphorylation (Mitchell's hypothesis) and other mitochondrial physiology interpretation and offered a different general theory based on the AI hypothesis (Ms. # 11).

b. I have also critically reviewed the current theory of active transport across bifacial types of cells (e.g., giant algal cells, skin and intestinal epithelia) and presented a new synthesis also based on the AI hypothesis. (Ms. #12).

There is also a considerable amount of research beyond those described. The urgency to submit this proposal made it impossible to present them all. However, if necessary, we can produce them at a later date.

In addition, I am also enclosing a copy of the flyer on the FONAR developed and now manufactured by Dr. Raymond Damadian. It is entirely possible that this will eventually replace the CAT-Scanner as a non-harmful and more powerful instrument. I am also enclosing a letter from Dr. Damadian, acknowledging that this development could trace its origin to the ONR supported work that we have done.

Publications

A. Published

1. G. N. Ling - "Ion and Water Transport: Experimental Design Defended", Trends in Biochemical Sciences 4:n134-n135 (1979)

2. G. N. Ling, C. Walton, and M. R. Ling - "Mg^{++} and K^{+} Distribution in Frog Muscle and Egg: A Disproof of the Donnan Theory of Membrane Equilibrium Applied to the Living Cells", J. Cell. Physiol. 101:261-278 (1979)

3. G. N. Ling and M. T. Tucker - "Nuclear Magnetic Resonance Relaxation and Water Contents in Normal Mouse and Rat Tissues and in Cancer Cells", J. Nat. Cancer Inst. 64:1199-1207 (1980)

4. G. N. Ling and W. Negendank - "Do Isolated Membranes and Purified Vesicles Pump Sodium? - A Critical Review and Reinterpretation", Persps. in Biol. & Med. 23: 215-239 (1980)

5. G. N. Ling, C. L. Walton, and T. J. Bersinger - "Reduced Solubility of Polymer-Oriented Water for Sodium Salts, Sugars, Amino Acids and Other Solutes Normally Maintained at Low Levels in Living Cells", Physiol. Chem. Phys. 12:111-138 (1980)

6. G. N. Ling, M. M. Ochsenfeld, C. Walton, and T. J. Bersinger - "Mechanism of Solute Exclusion from Cells: The Role of Protein-water Interaction", Physiol. Chem. Phys. 12:3-10 (1980)

B. In Press

7. G. N. Ling - "Underestimation of Na Permeability in Muscle Cells: Implications for the Theory of Cell Potential and for Energy Requirement of the Na Pump", Physiol. Chem. Phys. 12:#3 (1980)

8. G. N. Ling - "The Role of Multilayer Polarization of Cell Water in the Swelling and Shrinkage of Living Cells", Physiol. Chem. Phys. 12:#4 (1980)

C. Accepted for Publication

9. G. N. Ling - "Water and the Living Cell as seen from the Viewpoint of a New Paradigm", in International Cell Biology 1980-1981 published by Springer-Verlag, Second Int. Cong. on Cell Biol., W. Berlin

10. G. N. Ling, C. L. Walton, and M. M. Ochsenfeld - "A Unitary Cause for the Exclusion of Na^{+} and Other Solutes from Living Cells, Suggested by Effluxes of Na^{+}, D-Arabinose and Sucrose from Normal, Dying and Dead Muscles", J. Cell. Physiol.

D. Completed but not as yet Submitted for Publication

11. G. N. Ling - "Oxidative Phosphorylation and Other Aspects of Mitochondrial Physiology: A Reinterpretation in Terms of the Association-Induction Hypothesis Following a Critical Review of the Chemiosmotic Hypothesis"

12. G. N. Ling - "Active Solute Transport Across Frog Skin, Intestinal Epithelia, Kidney Epithelia and Other Bifacial Cell Systems According to the Association-Induction Hypothesis"

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Fox, S. W., R. J. McCauley, P. O'F. Montgomery, T. Takushima, K., Harada, and C. R. Windsor, in "Physical Principles of Biological Membrane", Snell, F., Walken, J. Iverson, G. J. and Lon, J. (eds.), Gordon and Breach Co., N.Y., p. 417-432 (1969)

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Moloff, B. L., S. P. Scordillis, C. Reynolds and H. Tedeschi, J. Cell. Biol. 78:99 (1978)

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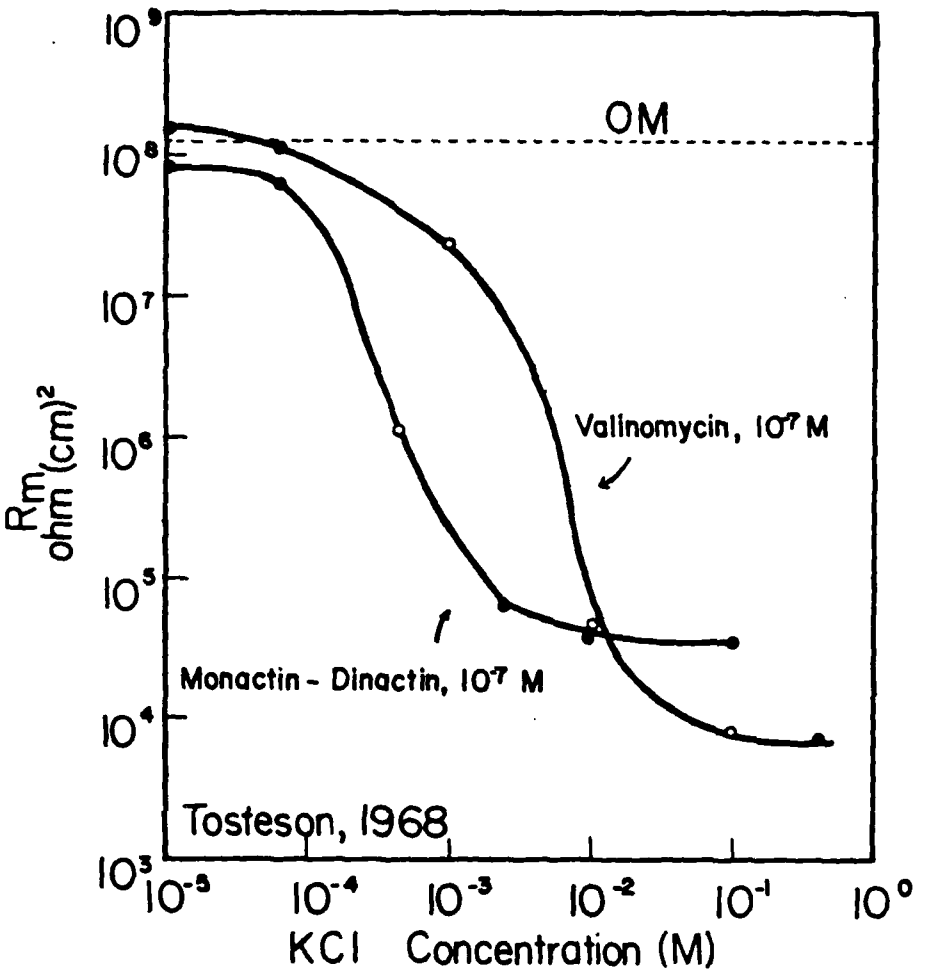

FIGURE 1

The powerful effect of valinomycin and monactin-dinactin at 10^{-7} M on the K^+ permeability of artificial phospholipid bilayer membrane. Membranes are prepared from extracts of sheep red cells. Figure taken from Tosteson (1968).

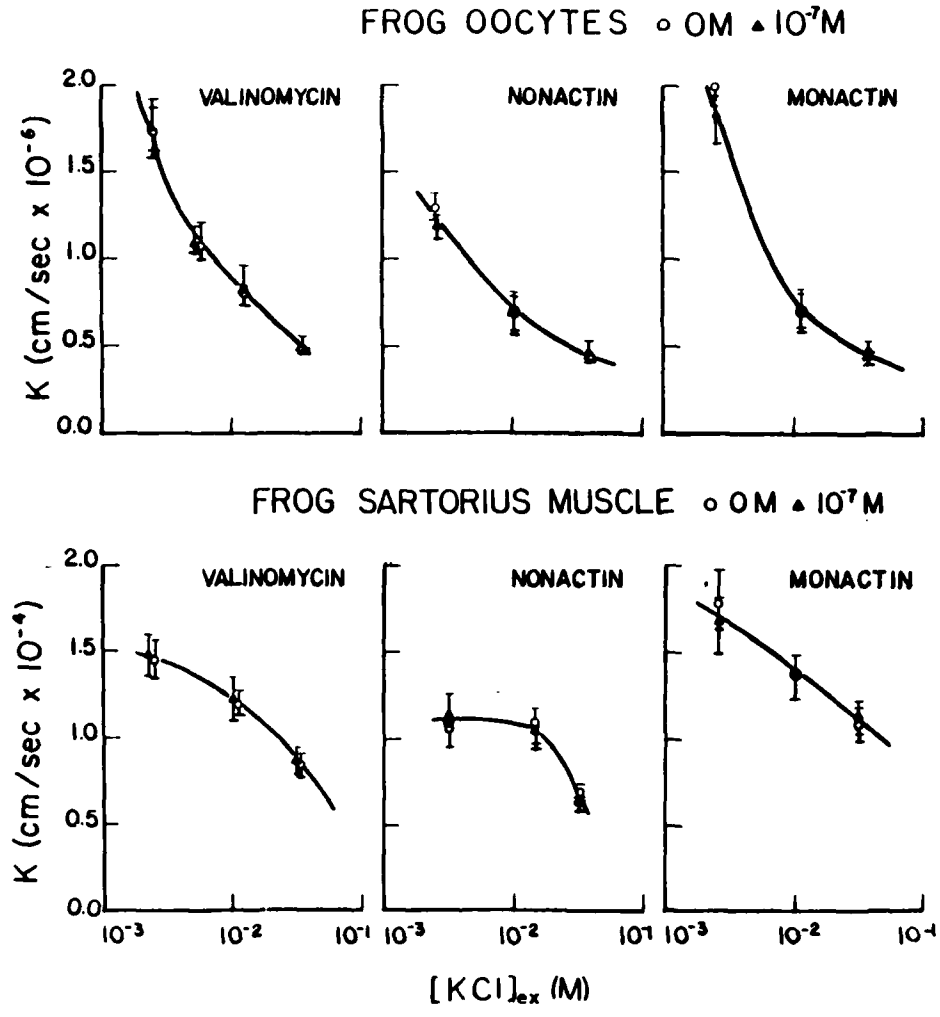

FIGURE 2

The lack of effect of valinomycin, monactin and nonactin at 10^{-7} M on the K^+ permeability of the membranes of living cells. K^+ permeability measured with ^{42}KCl. Abscissa is the KCl concentration in the bathing medium; ordinate is the permeability of the cell membrane to K^+-ion. Control (0); experimental (▲). Compare with Figure .

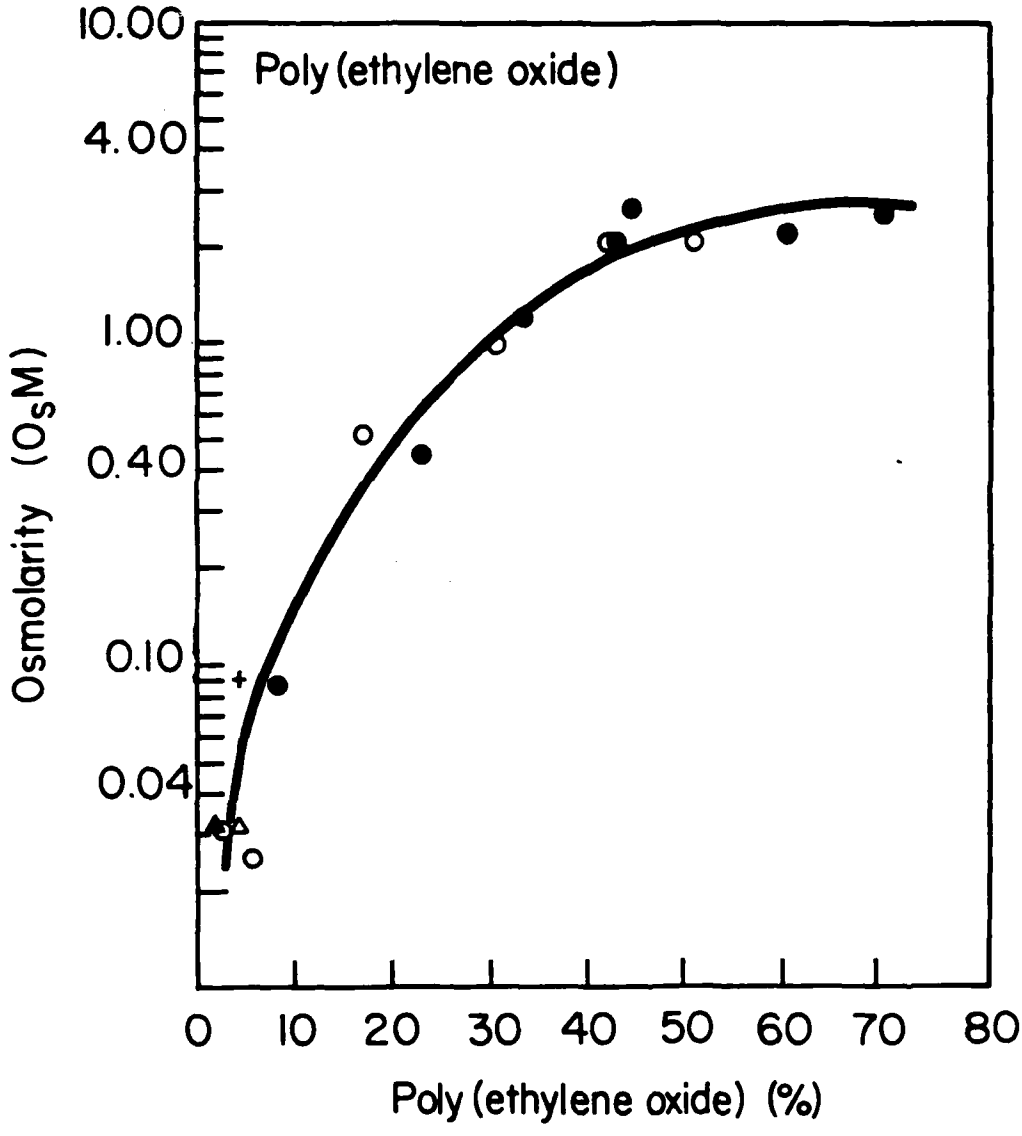

FIGURE 3

Osmotic activity of solutions of poly(ethylene oxide) (PEO), a non-charged polymer with a M.W. of 600,000. Each symbol represents the average value of data obtained from separate experiments. Abscissa represents PEO concentration in M/V percentage. Ordinate represents osmolarity

	Total $[Na^+]$ in sample	Measured Activity	Bound Na^+
Poly(Methacrylic Acid) PMA	1.03×10^{-1} M	$4.88 \pm .03 \times 10^{-2}$ M	5.42×10^{-2} M
Polystyrene Sulfonate TL-500	5.64×10^{-2} M	$2.44 \pm .01 \times 10^{-2}$ M	3.20×10^{-2} M

Polymer	Cation Added	Activity After Cation Added (M) 10^{-2}	Activity Before Cation Added (M) 10^{-2}	Displaced Na^+ (M) 10^{-2}	Initial Bound Na^+ (M) 10^{-2}	Percent of Bound Displacement
PMA	Arginine	$8.10 \pm .06$	$4.88 \pm .03$	$3.22 \pm .09$	5.42	59.4
	Guanidine	$7.76 \pm .02$		$2.88 \pm .05$		53.1
	Trizma HCl	$7.72 \pm .01$		$2.84 \pm .04$		52.4
	Triethanolamine	$7.32 \pm .04$		$2.44 \pm .07$		45.0
	Choline	$6.98 \pm .05$		$2.10 \pm .08$		38.7
	Tetramethylammonium	$6.79 \pm .03$		$1.91 \pm .06$		35.2
	Lysine	$5.74 \pm .05$		$0.86 \pm .08$		15.9
TL-500	Arginine	$5.34 \pm .01$	$2.44 \pm .01$	$2.90 \pm .02$	3.20	90.1
	Guanidine	$5.18 \pm .00$		$2.74 \pm .01$		85.6
	Triethanolamine	$5.14 \pm .03$		$2.70 \pm .04$		84.4
	Choline	$4.97 \pm .01$		$2.53 \pm .02$		79.1
	Tetramethylammonium	$4.93 \pm .01$		$2.49 \pm .02$		77.8
	Trizma HCl	$4.92 \pm .05$		$2.48 \pm .06$		77.5
	Lysine	$4.18 \pm .01$		$1.74 \pm .02$		54.4

TABLE I

Displacement of Bound Na^+ by Monovalent Cations

Each of the cations has been tested and shown to have negligible direct effect on the Na selective electrode. The "final" concentration of each of the displacing ions was 75 mM.

Research Proposal

Principal Investigator

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Research Proposal

General Aim:

Naval operations inevitably expose human beings to harsh and injurious circumstances. These encounters usually involve exposure of delicate living cells to adverse and unusual environments. Thus loss of blood in combat at once exposes tissue cells to the loss of oxygen and water; arctic operations expose tissue cells to freezing; severe concussion and injury-induced swelling exposes brain cells to extreme pressure; diarrhea deprives cells of both water and salt ions. The results of these and countless other harmful accidents or incidents is a loss of operational efficiency in naval combat or peaceful missions. The purpose of the present proposal is aimed at deciphering what are the focal points of injury and what factors may help the return to normalcy.

This five year project is divided into a total of 8 projects, each of which forms part of an integral whole to serve the general purpose depicted under General Aims.

Project I. The Role of "Structured Water" in Normal Intestinal Mucosal Cells as a Passive Barrier to the Massive loss of Water and Electrolytes and the Mechanisms Involved in Its Breakdown in Some Pathological Conditions Leading to Diarrhea.

A. Introduction

It is well known that the intestinal mucosa is not only the seat of active transport of water, salts, sugars, etc. into the healthy human body but also the focal point of malfunction in cholera and other diarrheal diseases involving rapid loss of body fluids. Far less well known is how normal intestinal mucosa serve this function.

In 1965 Ling presented at the Cholera Research Symposium sponsored by HEW a hypothesis directed specifically at the massive fluid loss induced by cholera infection (Ling, 1965): In brief, the normal barrier to massive fluid loss is the water in the mucosal cells, not ordinary water but water existing in the state of polarized multilayers. Cholera toxin causes a destruction of this normal barrier by interacting with the cellular proteins, which in their normal physiological state maintain the polarized multilayer state of the cell water. The result of this protein-toxin interaction is a depolarization of the water and a consequent loss of its semi-permeable properties. Polarized water referred to here is not limited to that at the microscopic cell membranes but includes that throughout the bulk of the cell.

While water in the state of polarized multilayers functions as a barrier to all solutes and water itself, its barrier effect can best be illustrated with NaCl, the main osmotically active component of the tissue fluids and blood plasma. No massive amount of water will pass through the mucosal barrier without a concomittant loss of NaCl for simple osmotic reasons. Thus the normal barrier functions of normal mucosa as well as its destruction by cholera toxin can, to a large measure reflect the mucosal cells ability to exclude NaCl. However, this is not to deemphasize the fact that polarized water is also a barrier to the movement of water itself.

It is implied that any other form of diarrheal diseases without the extensive physical destruction of the mucosal cells themselves may have a similar path-

ological mechanism. Confirmation of this hypothesis was achieved to a considerable extent by work supported by ONR as well as NIH by our laboratory as well as other's. Only a few examples need be cited here:

(1) Extensive chemical analysis of the cell membrane (does not include myelin) reveals that of its non-water components, proteins and not lipids are the predominant and constant components. (See Table P1 from Jain, 1972). Since all proteins hydrate (Ling, 1972) a constant components of the cell membrane is water.

(2) Polarized water model (cellulose acetate membranes) resemble living cells (inverted frog skin) much more closely than pure lipid membranes (in contrast to phospholipid membrane, which like cellulose acetate and many other nonlipid models of cell membranes also polarize water. (Ling, 1973)

(3) Intactness of the cell membrane is not essential for either the retention of water nor the osmotic behavior of the living cells (Ling and Walton, 1976).

(4) Interference with metabolism alters the state of water in living cells causing water loss from the poisoned cells under a centrifugal force whereas normal cells do not lose its water on centrifugation (Ling and Walton, 1976).

(5) Udall et al (1977) showed cholera enterotoxin on an intestinal loop preparation "can be explained by the theory of Ling". (Figure P1)

B. Specific Aim

To seek more precise understanding of the barrier effect of living cells to massive fluid loss such that eventually further improvements in the management of diarrheal diseases can be made.

C. Method of Procedure

In recent years, we have succeeded in confirming the polarized multilayer theory of "structured" water and in the course of these investigations we have been able to produce water in the state of polarized multilayers at will. This basic knowledge makes it possible to develop an experimental model that will permit us clearly to find out how water in this state, which has been proven to have reduced solubility for Na^+ salts, sugars, free amino acids, can serve as effective barrier to the fluid transfer under a hydrostatic or osmotic gradient.

Parallel with these developments of the model system and execution of experiments with these models, we shall also further investigate the barrier effect on living systems. In particular we would like to develop a system whereby the

effect of hydrostatic pressure differences on fluid transfer across mucosal membranes can be studied. Once a standard method has been established the membranes can then be subjected to various toxic materials including bacterial enterotoxins and metabolic poisons and various possible antidotes can then also be tried and studied.

D. Significance of Research

While the primary purpose of this project is to further our understanding of massive fluid loss in intestinal disorders, the barrier-effect of tissue cells is a physiological phenomenon of wide importance. Many major illnesses and injuries involve disturbance of this basic function: Fluid accumulation in the lungs after exposure to noxious gases and exudation of burn wounds are just two such examples. Clearly understanding and improvement in management of diarrhea can add broadly to strengthen the efficiency of naval operations.

Project II. Further Investigations Into Injury-induced Brain Swelling and Other Tissue Swelling

A. Introduction

Please see Page 3 to page 7 of Background Information.

B. Specific Aims

To achieve better understanding of the molecular events underlying tissue swelling in response to cold and injury and other noxious agents and eventually to provide better principles and guidelines toward the control of these pathological manifestations.

C. Methods of Procedures

Swelling phenomenon will be investigated using a variety of methods:

(1) Vapor equilibrium methods for H_2O equilibrium distribution will follow sterile procedures described by Ling and Negendank (1970): Sterile isolated tissues are exposed to environments of varying partial vapor pressure and the equilibrium water contents determined by drying in vacuo at low temperature or at 100°C in atmosphere pressure.

(2) Direct exposure methods for the equilibrium distribution are methods of long historical usage by immersing tissues in hyper-, iso- or hypotonic solutes. Again the key to reproducible results is sterility and the use of sucrose instead of salts to produce hypertonic solutions.

(3) Determination of extracellular space fluids may rely on one of the half a dozen methods introduced by this laboratory, including the poly-L-glutamate method of Ling and Kromash (1967), and the centrifugation method of Ling and Walton (1976)

(4) Determination of "Centrifugation Extractible Water" would be by the method of Ling and Walton (1975, 1976). This method potentially can separate water in its normal polarized multilayered state further of "depolarized" water following cell death or injury.

(5) Determination of ATP and other related compounds will rely on a modification of the firefly-enzyme method originally introduced by Strehler and McElroy. In our hands, the method permits accurate determination of ATP (as well as creatine phosphate and other intermediates) in a few milligrams of samples of drying tissues.

(6) Determination of chloride binding in salt-linkage splitting will rely on the efflux analyses described in the Progress Report attached.

D. Significance of Research

Since past research in this general area has been based on a concept that has been proven obsolete, the evolving of a basic understanding in terms of modern concepts is the only known way to achieve the purpose of understanding and controlling tissue swelling as a phenomenon which besides its broad significance in pathology causes death as in severe brain injury.

Part III. Thermal Transition, Heat Capacity and Other Thermal Phenomenon and Properties of Living Cells and Model Systems.

A. Introduction

It is well known that living cells can only tolerate a very narrow range of temperature variation. Human beings cannot tolerate a body temperature much in excess of 40°C; amphibians can tolerate even less, dying predictably at the body temperature of those of birds. Neither man nor amphibian nor birds can stand freezing for any length of time.

According to the association-induction hypothesis, this sensitivity to moderately high temperature reflects another basic property of the protein-water system of all living cells. As shown in Figure P2, the difference of a few degrees converts a perfectly normal frog muscle with full ability of excluding sucrose to one which has lost completely this property. Similarly at a temperature very close to that of frog muscle thermal transition, there is an entirely similar all or none

change in the state of water of two water polymer systems -- a transition from a state that shows pronounced ability to exclude sucrose, to one in which some 90% of the water has lost this ability (Figure p_3). This thermal transition, in terms of the association-induction hypothesis reflects a typical cooperative transition involving water. The free energy change involved in this transition of state of water from that under the long-range influence of the polymer to one no longer under its influence would yield important information concerning the basic difference of water in the state of polarized multilayers and in the state of normal liquid water. Since we are in possession of a collection of water-polarizing polymers with different chemical composition, the contribution of free energy change from the polymers themselves can be eliminated and that from the water extracted.

An equally important direction that this study can take is to provide a background for further analyses of the similarity as well as differences between the polarized water in living cells and in the model systems. Thus by careful blocking of metabolic reactions with poison, and by careful use of controls of disintegrated cells, etc., one can measure the heat change at the transition temperatures (e.g., frog tissues). By comparing with data from model system one can add major insights into the nature of cell water whose unique behavior have eluded biologists for so long.

B. Specific Aims and Methods of Procedures

To study the heat capacity and other thermal properties of water in the state of polarized multilayers in vitro and in vivo.

The heat capacity is another important property that can tell us about the structure of the water in living cells. To gain the maximum information, three elements are needed.

(1) An instrument capable of accurate measurement of specific heat over wide temperature range.

(2) A set of non-living model systems that has been proven to bring about long-range ordering of water; there must be enough variations in the detailed structure of these (polymer) model systems to permit sorting out the contribution from the polymers from the water.

(3) Knowledge about a variety of living forms and their physiological behavior to allow specific heat studies of their cell water, seperable from other

heat generating operations in the cells.

It is my belief with support from ONR, these requirements either have been or can be met in our laboratory.

The heat capacity is defined as the heat required to raise a system $1^{\circ}C$. If the system consists of a single substance, or a solution, and its weight is 1 g. then the heat capacity is called specific heat capacity or specific heat. Specific heat can be measured at constant volume (C_v) or constant pressure (C_p). For dealing with solid or liquids, we shall use C_p more conveniently. The key significance of heat capacity measurement is well illustrated by the fact that once we know the value of C_p, we can readily determine the other thermodynamic functions of the system at that temperature (enthalpy, entropy, and free energy): Thus, as an example, the difference in enthalpy of a substance at $T^{\circ}K$ and $0^{\circ}K$ ($H_T - H_o$) is given by

$$H_T - H_o = \int_o^T CpdT + \Delta Hpc \ , \qquad (1)$$

where ΔH_{pc} represents the sum of all enthalpy changes for phase transition occurring between 0 and $T^{\circ}C$. These thermodynamic parameters are essential for a deeper understanding of water in the state of polarized multilayers.

Another direction of research may be mentioned. As discussed above, one of the model polymer-water systems undergoes a phase transition at about 40° C ending with most of the water in the normal liquid state (Fig. P4). The enthalpy change of the system between say 0° C and 45° C can then be easily assessed calorimetrically. The data will permit us to test the hypothesis that in the state of polarized multilayers, the bonds between water molecules are stronger than in normal liquid water.

Heat capacity measurement from below 0° C-temperature to, say 100° C, of water in the state of polarized multilayers can also be studied in model polymer-water systems with varying water contents. A comparison of the heat capacity of this water with that of ice (I) and normal liquid water will offer further insight into the nature of the polarized multilayer state. In particular one can assess the "configurational contribution" to the heat capacity. In particular, these

studies can offer answers to several important but thus far elusive questions:

(1) Are there a substantial fraction of water in the model systems (and in non-metabolizing living cells) existing in a different physical state than the rest (i.e., two fraction hypothesis as often offered for NMR data interpretation).

(2) If, say a polymer-water system has a q-value of 40%, does this mean that only 60% of the water is in the different (e.g., polarized multilayer) state, while the rest is normal? Or are they uniformly in one polarized multilayer state? Again this can be assessed from the C_p values obtained at various water contents and ρ-values.

(3) Does a dead cell system lose all its water in the state of polarized multilayers or only the bulk of it?

C. Significance of Research

To provide basic knowledge essential for goals described under Project I and II.

Project IV. Further Study of the Donnan Theory of Membrane Equilibrium and the Alternative Model for Ion and Solute Distribution According to the Association-Induction Hypothesis.

A. *Introduction*

It is no exaggeration that Donnan's theory of membrane equilibrium published in the early twenties of this century represents the height of an intellectual development initiated by M. Traube's discovery of the near ideal copper-ferrocyanide semipermeable membrane and the postulation by W. Pfeffer of the membrane theory. Thus it would appear that Donnan, by inciting Gibb's basic thermodynamic theorem, provided the answer in a totally unified and coherent quantitative manner the solution of all the major cell phenomenon: cellular electrical potential; ionic distribution, and later with the aid of Procter and coworkers, also swelling and shrinkage. It was no wonder that the Donnan theory was widely celebrated by capable students of that time, including Jaques Loeb, Leonor Michaeles and many others. So that by now the Donnan membrane equilibrium is a household word taught and used as widely as many other physicochemical laws.

Yet as we have indicated in the Progress Report, there is little question that this theory is not correct. Extensive experimental studies unequivocally have disproved it. Once we have these experimental data on hand, it is not difficult to realize what had gone wrong. The theory was accepted too enthusiastically to permit an oversight - which in hindsight is unbelievably glaring. Donnan had inadvertently

violated the law of conservation of macroscopic electric neutrality! Once one realizes the limitation of this law, the the relation between electrical potential difference (or membrane potential) and ionic distribution can no longer hold. Ions can move only by either one of two mechanisms between the two macroscopic phases: (1) ion for ion neutral exchange; (2) ion-pair neutral migration. With this limitation in mind, one is more or less forced to accept the following equation for ion distribution: (a more detailed equation already fully derived, will be given elsewhere)

$$[P_i^+]_{in} = [P_i^+]_{\text{as ion pair}} + [P_i]_{\text{as free counterion}} + [P_i]_{\text{as adsorbed counterion}}$$

There is no dependency on the membrane potential and vice versa.

B. Specific Aims

To complete our investigation of Donnan's theory of membrane equilibrium and to test the alternative theory offered as a part of the association-induction hypothesis. In this alternative theory, ion distribution is independent of the electrical potential difference but is dependent on adsorption on sites, equilibrium distribution constants or q-value.

C. Methods of Procedure

The basic methods involve the study of ion and solute distribution across a membrane which is fully permeable to all other components except the "impermeable ion", usually in the form of a charged polymer of well-defined chemical structure and very large molecular weight (several hundred thousands and several million daltons). Ions are determined with isotopic method, atomic absorption spectroscopy and specific ion-sensitive electrodes.

D. Significance of Research

The basic quantitative relations governing Na^+ and other solute distribution are essential for our understanding of the barrier effect of living cells against massive fluid movement as well as tissue swelling.

Project V. Further Study of the Mechanism of Cellular Electrical Potential and the Detailed Mechanism of Selective Ionic Adsorption

A. Introduction

In the Progress Report, we have given a summary of major developments in our understanding of the cellular electrical potential. The Hodgkin-Katz ionic (HKI) model of the cell potential, a sequel to the Ostwald-Bernstein membrane poten-

tial model after these theories per se were shown to be untenable following the demonstration of permeability of the cell membrane to Na^+, long held to be totally impermeant in the membrane theory. The success-failure history of the HKI model led on one hand to further emphasis of a pump generated potential, the electrogenic pump hypothesis which would have made the successful part of the HKI model an entire mystery. On the other hand, it also led to the development of the surface-adsorption model as part of the association-induction hypothesis which, in contrast, to the electrogenic pump theory, fully incorporates all the successful elements of the HKI theory as well as those of the Ostwald-Bernstein theory.

According to the surface adsorption model, it is only fixed anionic sites on a microscopically thin layer of the cell surface that is responsible for the electrical potential. The difference in electrical potential sensitivity to K^+ and Na^+ at rest and during activity is not a matter of relative permeability; rather, it is a matter of selective adsorption. The extensive evidence in favor of this theory was reviewed by Ling (1978) and still more confirmation of a more generalized model by data given in the Progress Report. The quantitative success of this model has now provided new ways of understanding the key surface anionic sites as it is being modulated and controlled by drugs such as ouabain, cocaine, adrenalin, Ca^{++}, etc.

B. Specific Aims

(1) to study in living cells the control of selective ionic adsorption at the cell surface by cardinal adsorbents, including drugs of important clinical usefulness.

(2) to study selective ionic adsorption making use of the surface potential as a monitoring device.

C. Methods of Procedure

(1) Living Cells: Method of microelectrode measurements will follow essentially the procedure described by Ling and Gerard in 1949. Long term incubation, which is essential for these studies will follow the procedures described by Ling and Bohr in 1969.

(2) Model Systems: The basic procedures would be similar to those described by Ling (1967). Glass electrodes are coated with collodion or other polymers including proteins and the sensitivity of the surface potential to external ionic concentration provides the basis for calculating the selectivity according to the early version of the equation

$$\Psi = \text{Constant} - \frac{RT}{F} \ln \left(\sum K_i \, [P_i]_{ex} \right) ,$$

where K_i is the adsorption constant of the ith cation at concentration, $[P_i]_{ex}$, R, T, F have the usual meaning. To fix a protein onto the electrode surface, we may choose either (1) adsorption on a collodion matrix, (2) cross-linking or (3) imbedding in a non-charged gel matrix.

D. Significance of Research

In accordance with the association-induction hypothesis much of the controlling cardinal sites (receptor sites) of the cell reside on the cell surface. Their effects often manifest themselves as electrical potential charges. These proposed studies permit both an understanding of how these controls are exercised as well as quantitative measurements of these effects.

The rapid development of method of studying and later establishing the adsorbed state of the bulk of intracellular K^+ demands a more precise testing of the theory of selective adsorption. So far the equilibrium dialysis methods have been in general unsatisfactory for a theoretically understandable reason: that the c-value of the anionic sites in an isolated system tends to different values and then of great heterogeneity. The collodion electrode study exhibits a high degree of selectivity among the alkali-metal ions as shown in Figure P3 . We are hopeful that further extension of these earlier studies will add important information to this difficult field of ion specific adsorption on proteins.

The major departure of the association-induction hypothesis from the conventional membrane theory lies in specific adsorption. Specific adsorption rather than membrane permeability determines the cellular electrical potential. Selective adsorption rather than membrane permeability determines ion and water transport and this list can go on many steps further. For these reasons, the significance of this research is at once broad and specific, essentially for understanding of all phases of biomedical problems including those specifically spelled out under Project I and II.

Project VI. Osmometric Studies of Polymer Water and Polymer-Ion-Water Systems

A. Background Information

Osmotic pressure is an ancient concept deeply imbedded in all phases of biomedical thinking. It is interesting therefore to read in a standard modern classical treatise of physical chemistry (Textbook of Physical Chemistry by S. Glasstone, 14th printing, 1961, Van Nostrand, p. 651 to 681), "In the study of osmotic pressure the essential point to be borne in mind is that, for some reason connected with the presence of solute molecules, the partial free energy, chemical potential or activity of solute molecules is less in a solute than in a pure liquid; that is to say, the transfer of solvent from pure solvent to solute will result in a decrease of free energy" (p. 668). This statment clearly shows how much more do we need to know about this most basic phenomenon and the recent successful demonstration of water in the state of polarized multilayers - a state that can be established "on command" by proteins and other simpler macromolecules permits a new line of investigation using standard osmotic method.

As long ago as the beginning of the present century, the question has been raised, "Is an adsorbed ion osmotically totally inactive?" A. V. Hill believed that it is possible for an ion to be adsorbed and yet osmotically active. No proof has been given. Again the advent of new synthetic materials and ion-specific electrodes, and simple and accurate osmometers permit, for the first time, an investigation of this basic question - which is v e r y important to know more about as the theory of Donnan equilibrium which ONR supported work (see Progress Report) has disproven more than half a century after Donnan's proposition of the theory and in this period has permeated and served as the major pillar of the membrane theory of the living cells.

B. Specific Aims

To study (1) the osmotic activity of non-ionic polymer-water systems which shows pronounced ability to exclude solutes like Na salts, sugars and free amino acids, and (2) the osmotic activity of ionic polymer-counterion water system to determine if adsorbed ion is or is not osmotically active.

C. Methods of Procedure

It is now available in the market a vapor pressure osmometer which can measure the osmotic pressure in very small samples. It is thus planned to make measurement of the series of polymer-water systems which has now been established to have solute exclusion properties. These would include polyvinylpyrrolidone, polyethylene oxide, polyvinymethyl ether, with variations of polymer concentration, polymer orientation (by stirring), etc.

To measure the osmotic activity of adsorbed ions, we shall make measurement of counterion activity with ion specific electrodes in solutions of synthetic polymers with fixed anions (e.g., polystyrene sulfonate, polyanethole sulfonic acid, polymethacrylate, etc.) This activity is then compared with a chemically determined total counterion concentration to yield the concentration of free counterion and adsorbed counterion. The osmotic activity of the same systems are then studied with the vapor pressure osmometer which will yield essentially a concentration of osmotically active particles. Since the polymer molecular weight is great (mw ~ 200,000 to 1,000,000) its molarity is negligible. Thus the total osmotically active concentration must be entirely due to the counterion. When compared to the concentration of free and adsorbed ion measurement with the ion-specific electrodes we can reach a final conclusion regarding the question raised above: "Are adsorbed ions osmotically active?"

D. Significance of Research

There is no need to emphasize the great importance of osmotic pressure and osmosis either in tissue swelling phenomenon or in mass fluid loss phenomenon. Thus the basic gain from this research will be of importance to the long-range general goal stated earlier.

Project VII. Control of Metabolic Activity of Living Cells

A. Background Information

One of the most outstanding behaviors of normal healthy living cells is the exquisite control of the energization of work performance. Excited muscle cells can respond to functional activity by prompt activation of its energy supply mechanism. The major "power house" of eucaryotic cells resides in the mitochondria which as a rule are "suspended" in the cytoplasm and are thus not in direct contact with the cell surface. Thus a major missing link in our knowledge of metabolic control is the mechanism by means of which external perturbation or stimuli can control the mitochondrial activity with great precision and efficiency.

One set of observations that we believe may provide fundamental insight into the problem centers around what is commonly known as the "Solandt effect", i.e., the increase of heat production of muscle tissue in response to increased external K^+ concentration. However, the specific phenomenon of interest to us refers to the concomitant oxygen consumption rather than heat production - a finding in fact first observed by W. O. Fenn. In the Progress Report Section, we have already presented some earlier preliminary work we have performed.

B. Specific Aims

To study how extracellular perturbation (i.e., changes in the concentration of extracellular K^+, Ca^{++}, analgesic drugs like procaine, etc.) control the oxygen consumption of intact and traumatized muscle tissue. In particular how do these perturbations bring about the synchronized changes of glycolytic enzymes as well as mitochondrial activities.

C. Method of Procedure

The fundamental methods are the same as used before in this laboratory, employing the Yellow Spring Oxygen meter and the isolated frog muscles as the initial materials for study. To change the intracellular ionic composition of these cells, the long term preservation method of isolated tissues developed in this laboratory will be used.

D. Significance of Research

It is obvious that by now virtually every single biochemical reaction leading from glucose (and other food materials) to CO_2 and H_2O is known. Yet this high degree of biochemical sophistication contrasts with the profound lack of knowledge concerning how these mechanisms are functionally linked together to serve the function of the intact living cells. The present project is aimed at filling in on this major gap.

Project VIII. Blood-clotting and Related Phenomena: Further Investigation in the Control of the State of Bulk-Phase Water by Agents That React With the Water-polarizing Proteins

A. Background Information

One of the major advances in this laboratory, made possible by ONR support is the proof that bulk phase water can indeed be polarized in multilayers by proteins or other polymers which can provide a matrix of chains on which are found oxygen atoms separated from their nearest neighbor by a distance roughly equal to that of two water molecule diameters (see attached reprints Nos. 5 and 6), and that water in the state of polarized multilayers have reduced solubility for solutes in inverse relation to the solute's size and complexity. While these findings have confirmed a major postulation of the AI Hypothesis, there are other theoretical assumptions relating to this phenomena. Most important is the contention that proteins can control the state of water by conformation change and this conformation change is in turn controlled by agents (called cardinal adsorbents) that adsorb or otherwise react with certain key sites on the protein, thereby altering the electron distribution of the entire assembly. This aspect of the AI Hypothesis has not yet been experimentally tested and will be the subject of this project.

B. Specific Aims

To test the hypothesis that reagents that do not directly react with the protein backbone NHCO sites but react with side chain functional groups, can control the physical state of water.

C. Method of Procedure

Preliminary work described under Progress Report shows that vapor pressure osmometer can be used quantitatively to measure the state of multilayer polarization of water. It is our intention therefore to use this instrument further to study the effects of agents known to react specifically with side chain functional groups on the state of water. Two examples can be cited:

(1) Bovine serum albumin and hemoglobin undergo denaturation in acidic medium. By careful control of pH, the acidic groups involved can be titrated and the effect on the state of water assessed. Renaturation of the denatured proteins can al so be achieved by dialysis in neutral solutions and its effect similarly tested. The exp ted effect is (after due correction for the added acid) that the apparent osmotic activity should sharply rise with denaturation and just as sharply decrease with renaturation;

(2) Fibrinogen, is converted into fibrin on interacting with a very low n-centration of thrombin. We suggest that this process also involves a change of low degree of fibrinogen-water interaction (see enclosed reprint No. 5) to high degree of fibrin-water interaction, resulting in an abrupt shift of the apparent osmotic activity of the fibrin-water system.

D. Significance of Research

Blood clotting is the first step toward the healing of wounds. Further understanding of the basic mechanism in relation of a major component of the system, water, is clearly of interest to science in general and Naval research in particular.

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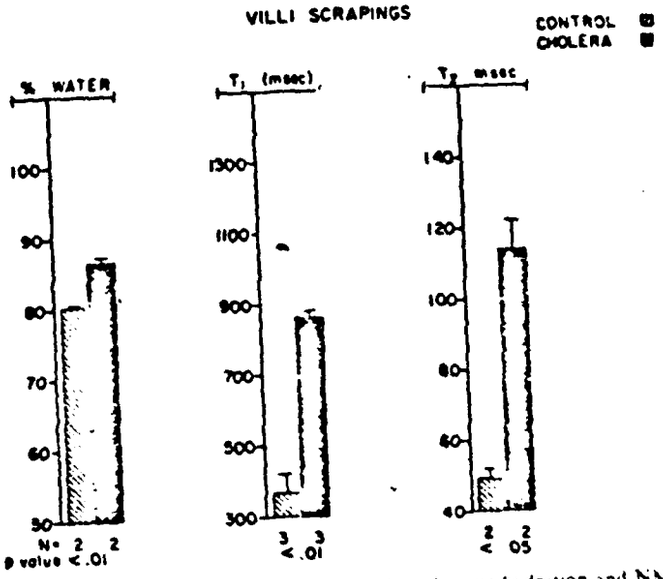
Mahendra K. Jain

COMPOSITION OF SOME TYPICAL PLASMA MEMBRANES

Type of Cell	*Protein (%)*	*Lipid (%)*
Ox brain Myelin	18-23	73-78
Human erythrocyte	53	47
Saccharomyces cerevisiae NCYC 366	49	45
Pseudomonas aeruginosa	60	35
Saccharomyces cerevisiae ETH 1022	37[a]	35
Bacillus megaterium	70	25
Micrococcus lysodeikticus	68	23
Rat muscle	65	15
Rat liver	85	10
Avian erythrocyte	89	4
Rod outer segment	40-50	20-40
Chlorophylls	35-55	18-37
Mitochondria (total membrane)	70	30
Mitochondria (inner membrane)	75	25
Sarcina lutea	57	23
Mycoplasma laidlawii	47-60	35-37
Bacillus spp.	58-75	20-28
Micrococcus lysodeikticus	65-68	23-26
Staphylococcus aureus	69-73	25-30

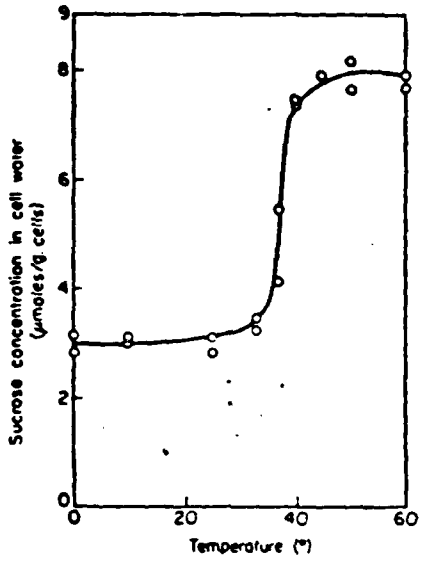
[a] Up to 27% mannan was found in the preparation.

TABLE P1



Effects of cholera enterotoxin on small bowel tissue hydration and NMR relaxation times in villi scrapings.

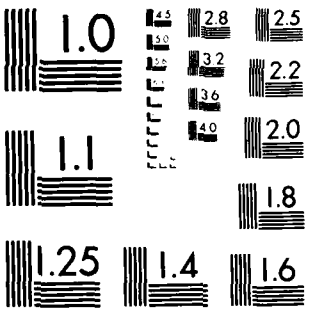
FIGURE P1

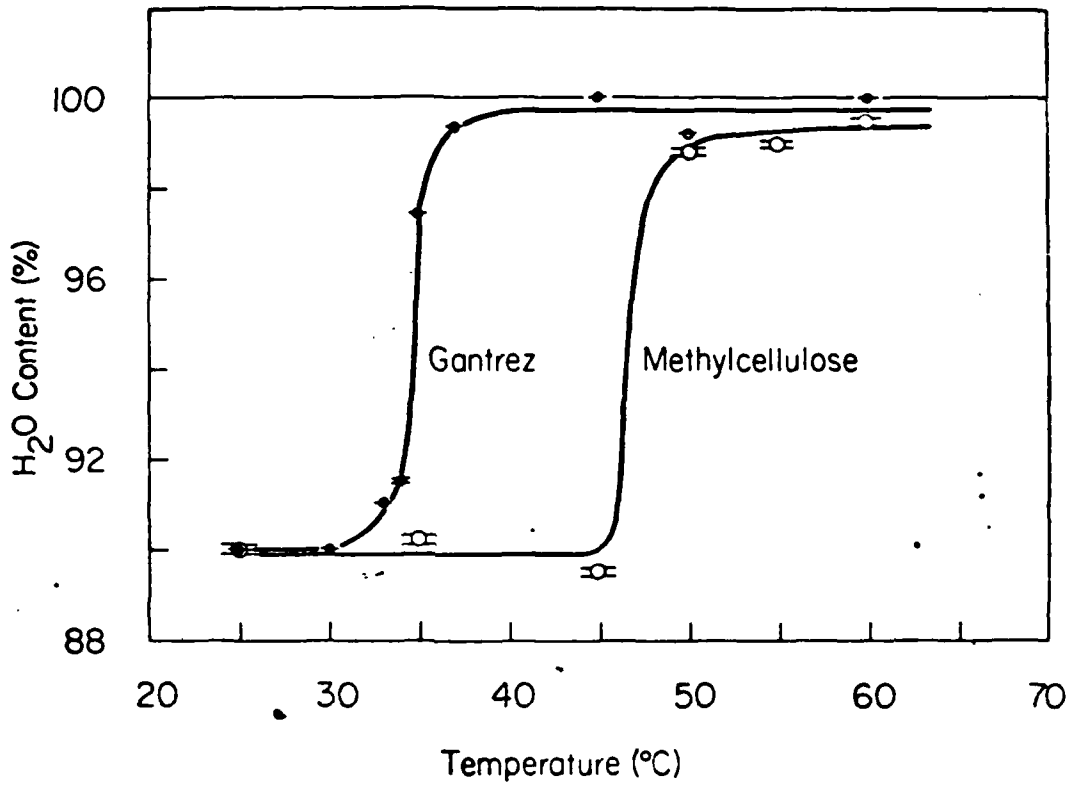


Effect of temperature on the equilibrium distribution of sucrose between the external medium and the cell water in frog sartorius muscles. The equilibrium concentration of glucose was 8·4 m*M*. Unpublished data of G. N. Ling and P. Shannon.

FIGURE 72

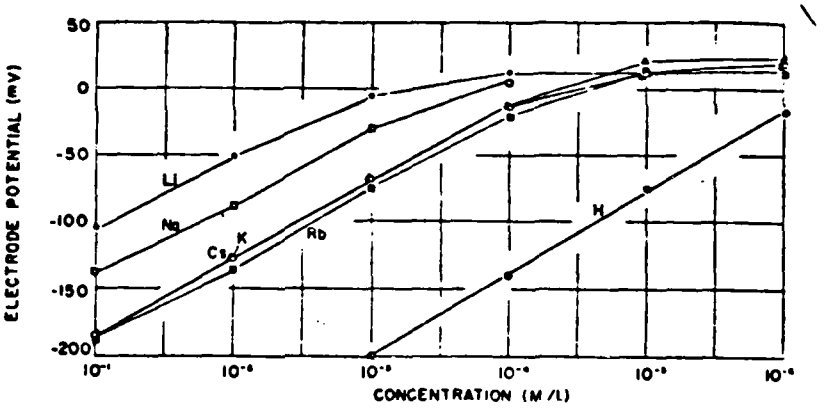
AD-A136 231 MOLECULAR MECHANISMS INVOLVED IN TISSUE SWELLING DUE TO 2/2
INJURY AND DUE TO..(U) PENNSYLVANIA HOSPITAL
PHILADELPHIA DEPT OF MOLECULAR BIOLOGY..
UNCLASSIFIED G N LING ET AL. 06 OCT 80 N00014-79-C-0126 F/G 6/1 NL





Cooperative transition at different critical temperatures of two macromolecule water systems. In each the water in the polymer system polarized in multilayers at a lower temperature suddenly becomes liberated turning into normal water at a specific temperature (the transition temperature). In response to the liveration of the bulk of the water the polymer separates out into a different phase leaving the bulk water with very little of the polymer. The ordinate indicates the percentage of water in the bulk phase. At below critical temperatures the water content in these cases is about 90%. After the transition temperature the water contents approach 100%.

FIGURE P3



Monovalent cation sensitivity of a collodion-coated glass electrode. The potential is considered positive if the outside solution is positive with respect to the collodion-glass phase.

FIGURE P4

Budget Justification
December 1, 1979 to November 30, 1981

Our total budget for the year 1978-1979 was $97,769. The proposed for the 1979-1980, 1980-1981 are higher at $115,490 and $113,653.

This marked increase for the projected budget was entirely due to the decision of policy-makers of the Pennsylvania Hospital concerning the overhead. Thus the overhead for 1978-1979 was only $13,295; for the coming two years at the Hospital's request it has gone up to $35,170 and $37,794 respectively, an increase of over $20,000 for each year! The total direct costs on the other hand are:

	Direct Cost
1978-1979	$ 84,474
1979-1980	$ 80,140
1980-1981	$ 75,589

Thus in spite of sky-rocketing inflation, we have actually been requesting lesser and lesser direct costs for each year. To achieve this we have to fire one of our research assistants.

Detailed Budget Justification
December 1, 1979 to November 30, 1981

A. Research assistants: We have three major projects more or less the same size. The other two each paid for 42% of the principal investigator's salary, which ONR only pays 16%. This imbalance is roughly made up by ONR paying for another research assistant. Therefore in truth ONR does not support many research assistants; it supports only one.

The fringe benefits are those required and decided on by the Hospital administration.

B. Capital Equipments:

(1) Vibrating reed electrometer: This is the electrometer with the highest input impedance 10^{16} ohms and it was with this instrument our original work on surface electrical potential was done at the Eastern Pennsylvania Psychiatric Institute (See Project V of Research Proposal). As the high impedance of the special glass electrodes cannot be measured by other instruments we have, this item is essential for Project V.

(2) Strip chart recorder: is an suailiary instrument for the vibrating reed electrometer.

(3) Wescor vapor pressure osmometer: is essential for Project VI for the measurement of osmotic activities.

C. Other

Service maintenance costs are extremely high. These are shared among all three projects.

D. Indirect Costs

For the past 16 years the Pennsylvania Hospital has charged a 20% overhead. This year they demanded to bring it up to the same level arrived at with NIH, 50%. Again we have no choice. The Hospital will not sign our application without requesting this overhead.

Office of Naval Research

Proposed Budget - December 1, 1979 to November 30, 1981

		1st Year		2nd Year	
1. Personnel					
Principal Investigator	16%	$ 7,735		$ 8,431	
Research Assistant	100%	11,712		12,766	
Research Assistant	100%	10,698		11,661	
Secretary	24%	2,727		2,972	
Summer Students	100%	8,000		8,000	
Laboratory Aide	100%	9,063		9,879	
			(49,935)		(53,709)
Fringe Benefits Social Security, Medical Insurance, Tuition Reimbursement		6,105		6,780	
Total Personnel Costs			56,040		60,489
2. Capital Equipment					
Vibrating Reed Electrometer		4,400		-	
Strip Chart Recorder		1,900		-	
Vapor Pressure Osmometer (Wescor, 51100BXR) and accessories		3,500		-	
Total Equipment Cost			9,800		0
3. Supplies					
Chemicals, biological culture media		2,500		2,600	
Radio chemicals		1,200		1,300	
Animals plus food and bedding		800		850	
Glassware including NMR tubes and scintillation counting vials		900		900	
Gases (pure oxygen, nitrogen, carbon dioxide, helium and mistures, liquid nitrogen, acetylene)		850		875	
Glassware cleaning and sterilization supplies		450		475	
Electron microscope supplies		500		500	
Miscellaneous supplies including filter paper, Parafilm, dissecting instruments		800		850	
Office supplies including data paper, graph paper, recorder paper		800		850	
Total Supplies Cost			8,800		9,200
4. Travel			400		400
5. Publication Costs					
Page costs, photoprints, illustrations, reprints			1,000		1,000
6. Other					
Service maintenance contracts and repair on instruments including AEI-6B electron microscope, Jelco Minimar NMR Spectrometer, Spin-Lock pulse NMR Spectrometer, beta-scintillation spectrometer, gamma-counting systems, DSC-2 Scanning Calorimeter, centrifuges, balances, spectrophotometers		2,800		3,100	

Proposed Budget - Dec 1, 1979 to Nov 30, 1981 (Page 2 of 2 Pages)

	1st Year		2nd Year	
6. Other (Continued				
Disposal of radioisotopes and osmium; detectopm badge service	600		700	
Journals, books, scientific articles	500		500	
Fees for NRC license, meetings, services, conferences, etc.	200		200	
Total Other Costs		4,100		4,500
Total Directo Costs		80,140		75,589
Indirect Costs (Overhead 50%)		35,170		37,794
Total		115,310		113,383

Justification

Request for Expansion of Present Contract - December 31, 1981 - November 30, 1984

Preamble

"Navy has a vital interest in the health, the innovativeness and the aggressiveness of American science... the present and future generations of naval officers and enlisted personnel must be technically trained, but they must be receptive and enthusiastic in matters of science if we are to realize the operational efficiency and the leverage that science offers and that we must emphasize if we if we are to maintain our present position to the other navies of the world."

(RADM A. J. Baciocco, Jr., USN
Chief of Naval Research in "The Future of American Science"
Naval Research Rev. 32:2 (1980)

- - -

"In almost every country, universities again became significant instrumentalities for the conduct of scientific research. But in Germany, France, Japan and the East European nations, powerful freestanding institutes are the principal loci for front-rank research..."

(Phillip Handler, President, National Academy of Sciences
Presented at the first Admiral Charles H. Davis Lecture)

- - -

"Authorities, disciples, and schools are the curse of science: and do more to interfere with the work of the scientific spirit than all its enemies."

(T. Huxley: reported to have said, two years before his death)

- - -

Background

Unlike physics and despite some phenomenal achievements, biology is still at its infancy. At this stage of development biology can make some, but relatively limited , contributions to the welfare of mankind in general and Naval operations in particular. However, once biology has reached maturity, its contribution would be beyond imagination. In other words, biology is not unlike a baby golden goose - not much meat even if you cook it now but if allowed to mature will one day produce an endless number of golden eggs.

Scientific research in general and biological research in particular resemble at once a crossword puzzle and a fox hunt. It is like a crossword puzzle because there is only one solution and all the individual pieces must fit together and that uncoordinated piecemeal attack, in the long run would lead to nowhere. It is also like a fox hunt because if the first direction taken is wrong, subsequent steps following that general direction no matter how sophisticated and "advanced" they may look could only be more mistakes.

Research in biology and medicine covers a vast terrain. Many scientists must be engaged in the efforts and specialization and division of labor is at once inevitable

and necessary. However, serious problems have developed because specialization occurs long before foundation concepts, which if correct would provide coherence among the specialists, have not been clearly established. It then became much easier and safer for a research scientist to restrict his effort to a highly specialized area. All he needs to do is to work in harmony with the few people (peers) in this highly specialized field and receive their endorsement and promotion. It is also much easier for a journal editor to accept his highly specialized paper in a specialized journal and to obtain high rating for grant proposals. It is, as if it were, dividing the crossword puzzle into many small sectors has minimized conflicts and maximized harmony.

The truth is if long continued, making a good and gracious living for all involved would have substituted for the real purpose of the research - which is to find the one and only one solution of the entire crossword puzzle - the truth. We are reminded how terrible this can become when we recall that other equally gracious people are sent to jail for having done a similar thing - using public money designated for purposes to serve the public is diverted into enriching one's own happiness.

Thus, by all criteria, something must be done to prevent this situation from getting worse. In my view, the key lies in the encouragement of fundamental research of the broadest significance that would eventually provide the coherence among all areas of specialization and through it the ultimate understanding that would profit all mankind.

I am happy and proud to tell you that in fact the Office of Naval Research has been doing just that for the last fifteen years. The proposal we are submitting is intended to continue this important enterprise.

The New Concept of the Living Cell: The Association-induction Hypothesis

The basic unit of all life is the living cell. Therefore in biology and medicine nothing could be more fundamental than that pertaining to the question of what is a living cell in the physico-chemical sense.

The conventional view, is that a cell is in essence a sacful of solution containing the right assortment of small and big molecules and surrounded by a submicroscopic cell membrane in which are found a battery of many pumps. It is the continual activity of these pumps that keeps the levels of cellular contents different from those in the environment. Thus in this theory pumping is living.

Shortly after I finished my Ph.D. at the University of Chicago in 1948, I began to encounter findings contrary to this view and I began to have serious doubts. These doubts eventually persuaded me to investigate, with the aid of a new tool, the (Ling-

Gerard) microelectrode, the minimum energy need of one pump, the Na pump. The efforts lasted over a number of years. The complete data were not presented until 1962. The conclusion was that the Na pump would consume at least 15 to 30 times as much energy as the cell commands.

This conclusion was repeatedly confirmed by other laboratories and no serious challenge to our results ever appeared. Three remedial postulations to keep the pump concept afloat were in turn all experimentally disproven.

Besides providing evidence against the membrane-pump theory, in 1962 I also offered an alternative new theory of the living cell, the Association-induction Hypothesis (AI Hypothesis) in the form of a monograph entitled "A Physical Theory of the Living State" (For a brief description of this theory and its evidence, see attached MS # 9)

It was two years later after the publication of my first monograph that I obtained support for the first time from ONR for a project in connection with the blood preservation - because the AI Hypothesis offered a more reasonable explanation for the role of glycerol in long-term blood preservation. This was the beginning of repeated renewals of support for both basic research and those more directly connected with Naval needs, like tissue injury, cholera, and other problems more immediately connected with Naval operations.

A very large amount of work has been accomplished, leaving no question that the membrane-pump theory is not tenable and that the AI Hypothesis is supported in virtually all of its basic concepts: As examples, we may mention that practically all K^+ in muscle cells is not free as in the membrane theory but is in an adsorbed state as the AI Hypothesis predicts and that proteins when existing in an extended conformation polarize water in deep layers and that it is reduced solubility for Na^+ in the polarized cell water rather than pump that is the cause of the low levels of Na^+ and other solutes in living cells again as predicted by the AI Hypothesis.

Thus through the continued and uninterrupted support of the ONR (and NIH), the first stage of a major paradigm transition is near completion (for a more detailed account of this transition see MS # 9).

Justification Proper

In the last 20 years we have also willy-nilly developed a new, free-standing research institute not unlike the kind Dr. Philip Handler found in many European countries and Japan. Members of this institute are full-time employees, devoted to a single purpose - research. None of us has duties other than those directly concerned with research. This degree of concentration and intensive efforts has been and is absolutely essential for the kind of efforts we are engaged in - efforts dealing with

the entire "crossword puzzle" on many research fronts all at once.

Equally important, we have much more space than most university professors and therefore capability to house facilities to conduct research over a wide area and the maneuverability to choose the most profitable approach at all times.

In addition we also have assembled a highly capable crew whose achievements are partly presented in the Progress Reports.

The proposed work is part of an effort to broaden and to develop in depth the AI Hypothesis and to extend this basic concept to other areas of research efforts which so far have been based on only the obsolete membrane-pump theory. Again within limits, we shall attempt to help in solving more practical problems of direct interest to the Navy.

To build bridges to both contemporary and future generations of students, I am beginning to put together another monograph. This is urgently needed because my first monograph (long out of print) was presenting a theory. The large amount of experimental testing and supportive evidence are published in many journals, not all easily accessible. As an integral part of this effort, I have been reading extensively and so far finished three major papers. One, written in coauthorship with Dr. William Negendank deals with vesicular work, and is already in print./ (see MS #4) The second one attempts to reinterpret the major findings in mitochondrial physiology in terms of the AI Hypothesis. The third, did the same with real active transport across kidney epithelium, frog skin, etc. Both are in the final draft state and are enclosed as MS # 11 and 12.

The small institute we have has no permanent endowment. All expenses have been derived from public grant agencies. As of now the Office of Naval Research, the Institute of General Medical Science (GMS), and the National Cancer Institute (NCI) of NIH each share 1/3 of the total expenses including 1/3 of the principal investigator's salary. Both the grant from GMS and NCI are five year grants. This long-term support eliminates a great deal of time spent in writing detailed proposals and in many other ways helps the overall research efforts. Our request for support for a long period of time is based on the same needs.

In conclusion, I would like to cite a parable that I used on a number of occasions. The parable of Queen Victoria's transistor radio - more as a reminder than anything else.

Suppose we can send to Queen Victoria a transistor radio through a time machine and that she is vastly entertained by the little music box. But one day the radio fell and broke into little pieces. Bereaved and unhappy, she vowed to repair the radio regardless of cost, money and manpower. Could she then succeed in the repair?

We can answer very positively, No. At Queen Victoria's time there was not enough fundamental knowledge of physics. Once physics has advanced to a mature state however,

a transistor radio can be fixed for a few dollars.

At this date and age, biology is about in the same stage of development as physics at Queen Victoria's time. Any human illness, malfunctions can receive some paliatory treatments by careful search, but true solutions and cures can only come after biology has achieved some degree of true maturity. It is in this task of nurturing this growth of fundamental biology, that ONR, especially the Biophysics Section, has made invaluable contribution to mankind. At the end of the Progress Report I cited the exciting development by Dr. Raymond Damadian of FONAR - an instrument of great potential for diagnosing injury and illness, an instrument that is already in production. In the appended copy of his letter Dr. Damadian traced its development to the work accomplished in this laboratory while supported by ONR (and NIH). More useful devices and results would inevitably come as basic biology marches on.

Justification

for Requested Budget for Expansion Years - December 31, 1981 - November 30, 1984

Our present contract budget allows direct costs for the 1979-1980 year of $80,140, and for the 1980-1981 year of $75,589.

Our expansion proposal for years covering December 31, 1981 through November 30, 1984 requests direct costs of $101,784 for third year, $98,649 for fourth year and $113,144 for fifth year.

The greatest part of this increase is for the purchase each year of one piece of equipment to replace an instrument that has given us daily service for 10-15 years. Breakdowns and repair service costs have increased dramatically; obtaining replacement parts has become difficult, too impossible. The replacement equipment requested:

High speed refrigerated centrifuge: essential to separate components of biological materials (e.g., red blood cells and ghosts, mitochondria, myosin) as well as many organic and inorganic suspensions. Centrifugation is a first step in many procedures for analysis of tissues. Present piece purchased in 1965.

Gamma counting system: Present instrument was purchased in 1966 and it is impossible to get replacements for mechanical parts of system. Many lab. studies (e.g., permeability and swelling studies) involve radioactive tracer techniques).

Atomic adsorption spectrophotometer: Needed to analyze ionic concentrations of biological tissues and organic and inorganic material studies. A.A. allows a simple method of measuring all of the major and trace elements of biological importance.

The remaining increase represents an average 7-8% rise in remaining categories. With the present double-digit inflation rate we feel that this represents a substantial cutback and economizing for the laboratory.

Office of Naval Research

Proposed Budget - December 1, 1981 - November 30, 1984

		3rd Year		4th Year		5th Year	
1. Personnel							
Principal Investigator	17%	$ 9,022		$ 9,744		$10,525	
Research Assistant	100%	13,207		14,276		15,422	
Research Assistant	100%	12,003		12,980		14,030	
Secretary	25%	3,569		3,860		4,175	
Laboratory Aide	100%	11,845		12,812		13,857	
Summer Students	100%	8,000		8,000		8,000	
			(57,646)		(61,672)		(66,009)
Fringe Benefits							
Social Security		3,619		3,898		4,198	
Medical Insurance		3,769		4,259		4,812	
Tuition Reimbursement		500	(7,888)	500	(8,657)	500	(9,510)
Total Personnel Costs			65,534		70,329		75,519
2. Capital Equipment							
Perkin-Elmer Atomic Adsorption Spectrophotometer Model 5000		20,400					
Sorvall RC2-5B Superspeed Refrigerated Centrifuge				11,420			
Packard Instrument Gamma Counting System						19,500	
Total Equipment Cost			20,400		11,420		19,500
3. Supplies							
Chemicals, biological culture media		2,300		2,400		2,550	
Polymers, resins		1,000		1,050		1,100	
Radiochemicals		1,000		1,100		1,225	
Glassware, plasticware including NMR tubes		1,100		1,200		1,300	
Gases (pure oxygen, nitrogen, acetylene, carbon dioxide, helium, liquid nitrogen, and mixtures & demurrage)		900		950		980	
Animals plus food and bedding		1,000		1,100		1,225	
Glassware cleaning and sterilization supplies		500		525		560	
Miscellaneous supplies, including filter paper, Parafilm, dissecting instruments, dialysis tubing		900		950		1,000	
Office supplies		400		425		460	
Data paper, graph paper, recorder paper		500		550		600	
Total Supplies Cost			9,600		10,250		11,000
4. Travel			500		500		500
5. Publication Costs			1,250		1,350		1,500
Page charges, photoprints, illustrations, reprints							
6. Other							
Service maintenance contracts and repair on instruments including:		3,000		3,200		3,450	

Proposed Budget - December 1, 1981 - November 30, 1984 (Pg. 2 of 2 Pgs.)

	3rd Year		4th Year		5th Year	
6. Other (Continued)						
DSC-2 scanning calorimeter, Jelco Minimar NMR spectrometer, Spin-Lock pulse NMR spectrometer, Beta-scintillation spectrometer Gamma Counting System, balances, centrifuges, spectrophotometers, AEI electron microscope						
Journals books scientific articles	600		650		650	
Radioactive wast disposal, detection badge service, licensing, etc.	500		550		600	
Library memberships	50		50		75	
Fees for conferences, meetings, seminar services	150		150		150	
Construction of special glass & laboratory apparatus	200		200		200	
Total Other Costs		4,500		4,800		5,125
Total Direct Costs		101,784		98,649		113,144
Indirect Costs (Overhead 50%)		40,692		43,614		46,822
Total		142,476		142,263		159,966

Pennsylvania Hospital
Philadelphia, Pennsylvania 19107

CONTINGENT FEE

(a) He ☐ has, ☒ has not, employed or retained any company or persons (other than a full-time bona fide employee working solely for the offeror) to solicit or secure this contract, and (b) he ☐ has, ☒ has not, paid or agreed to pay any company or person (other than a full-time bona fide employee working solely for the offeror) any fee, commission, percentage, or brokerage fee contingent upon or resulting from the award of this contract: and agrees to furnish information relating to (a) and (b) above, as requested by the Contracting Officer. (Interpretation of the representation, including the term "bona fide employee", see Code of Federal Regulations, Title 41, Subpart -1.5).

EQUAL OPPORTUNITY

(a) He ☒ has, ☐ has not, participated in a previous contract or subcontract subject either to the Equal Opportunity clause herein or the clause originally contained in section 301 of Executive Order No. 10925, or the clause contained in Section 201 of Executive Order No. 11114; that he ☒ has, ☐ has not, filed all required compliance reports; and that representations indicating submission of required compliance reports, signed by proposed subcontractors, will be obtained prior to subcontract awards. (The above representation need not be submitted in connection with contracts or subcontracts which are exempt from the equal opportunity clause.)

(b) The bidder (or offeror) represents that (1) he ☒ has developed and has on file, ☐ has not developed and does not have on file, at each establishment affirmative action programs as required by the rules and regulations of the Secretary of Labor (41 CFR 60-1 and 60-2) or (2) he ☐ has not previously had contracts subject to the written affirmative action programs requirements of the rules and regulations of the Secretary of Labor. (The above representation shall be completed by each bidder (or offeror) whose bid (offer) is $50,000 or more and who has 50 or more employees)

STATEMENT REGARDING ACQUISITION OF FACILITIES

The contractor, represented by an executive corporate official, or his equivalent in non-corporate entities, either expresses in writing his unwillingness or financial inability to acquire the necessary facilities with his resources.

Harry E. Heston (date)
Vice President
(Official Authorized to Sign for Institution)

PENNSYLVANIA HOSPITAL
The Nation's First Hospital · Founded 1751

DEPARTMENT FOR SICK AND INJURED
EIGHTH AND SPRUCE STREETS
PHILADELPHIA, PENNSYLVANIA 19107
TELEPHONE (215) 829/

November 10, 1980

Mrs. Farrington
Office of Naval Research
Code 613 KF
800 N. Quincy St.
Arlington, VA 22217

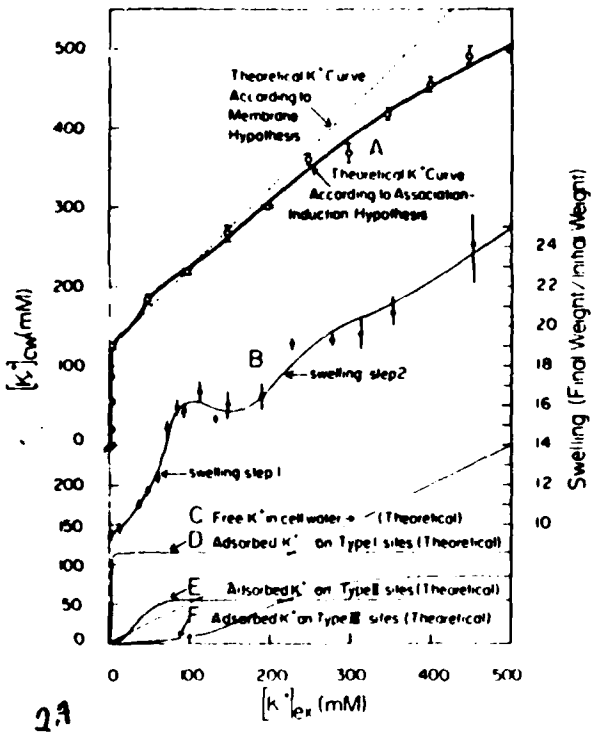
Dear Mrs. Farrington,

The hospital is in a circumstance where it is not in a position to pay for equipment used for research in Contract # N00014-79-C-0126. Without this equipment the project cannot be continued as orignally awarded. Please accept this as a poverty statement for Pennsylvania Hospital.

Sincerely,

Harry Heston
Vice-President

HH/db



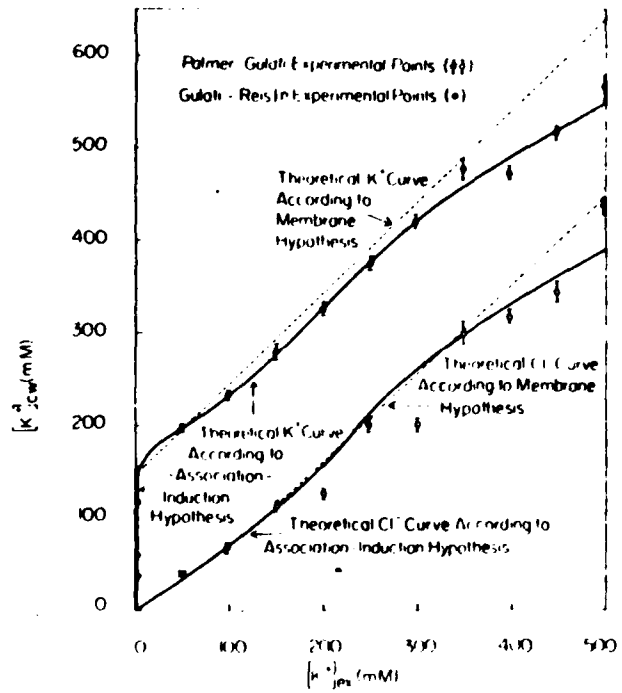


FIGURE 2A

Fig. 4 (left). Potassium concentration in frog muscle cells in the presence of 91 mM external NaCl. (○) New data on K^+ accumulation confirming those of Palmer and Gulati (*1*); (●) new data on muscle swelling, and (⊙) old data of Ling and Bohr (*8*) on K^+ accumulation. Curve A is a theoretical curve derived from the explicit form of Eq. 1 (*3*), which is resolvable into components shown as curve C [free K(Cl)], curve D (type I adsorption), curve E (type II adsorption), and curve F (type III adsorption). The contribution of type I sites was determined from the results of previous studies (*7-9*); those of type II and type III sites were estimated from curve B, which records the two-step swelling of frog muscles under conditions similar to those of curve A, except that a low external NaCl concentration of 30 mM was used (*19*). The q value used to obtain curve C was 0.5. Other numerical values used to obtain curves D, E, and F, respectively, were $[F]_t$ = 122, 55, and 85 mM; K_1 = 1.35, 35, and 185 mM; and $-\gamma/2$ = 0.54, 1.36, and 0.91 kcal/mole. For all data points the lengths of the error bars represent twice the standard error based on four or more determinations. The dashed straight line, predicted on the basis of the membrane theory as given by Palmer and Gulati (*1*), intercepts the ordinate at about 150 mM. Fig. 2 (right). Potassium and chloride in frog muscle cells. The experimental points are from Palmer and Gulati (*1*) and Gulati and Reisin (*10*) as indicated. Solid curves were derived from the explicit form of Eq. 1 (*3*). Dashed lines were derived on the basis of the membrane theory. The numerical values used to obtain the theoretical curves for K^+ were q = 0.5 for curve C and, for curves D, E, and F, respectively, $[F]_t$ = 150, 12, and 120 mM; K_1 = 1.0, 28, and 210 mM; and $-\gamma/2$ = 0.60, 1.36, and 0.91 kcal/mole. The theoretical curve of Cl accumulation is equal to that for K^+ accumulation minus type I adsorption.

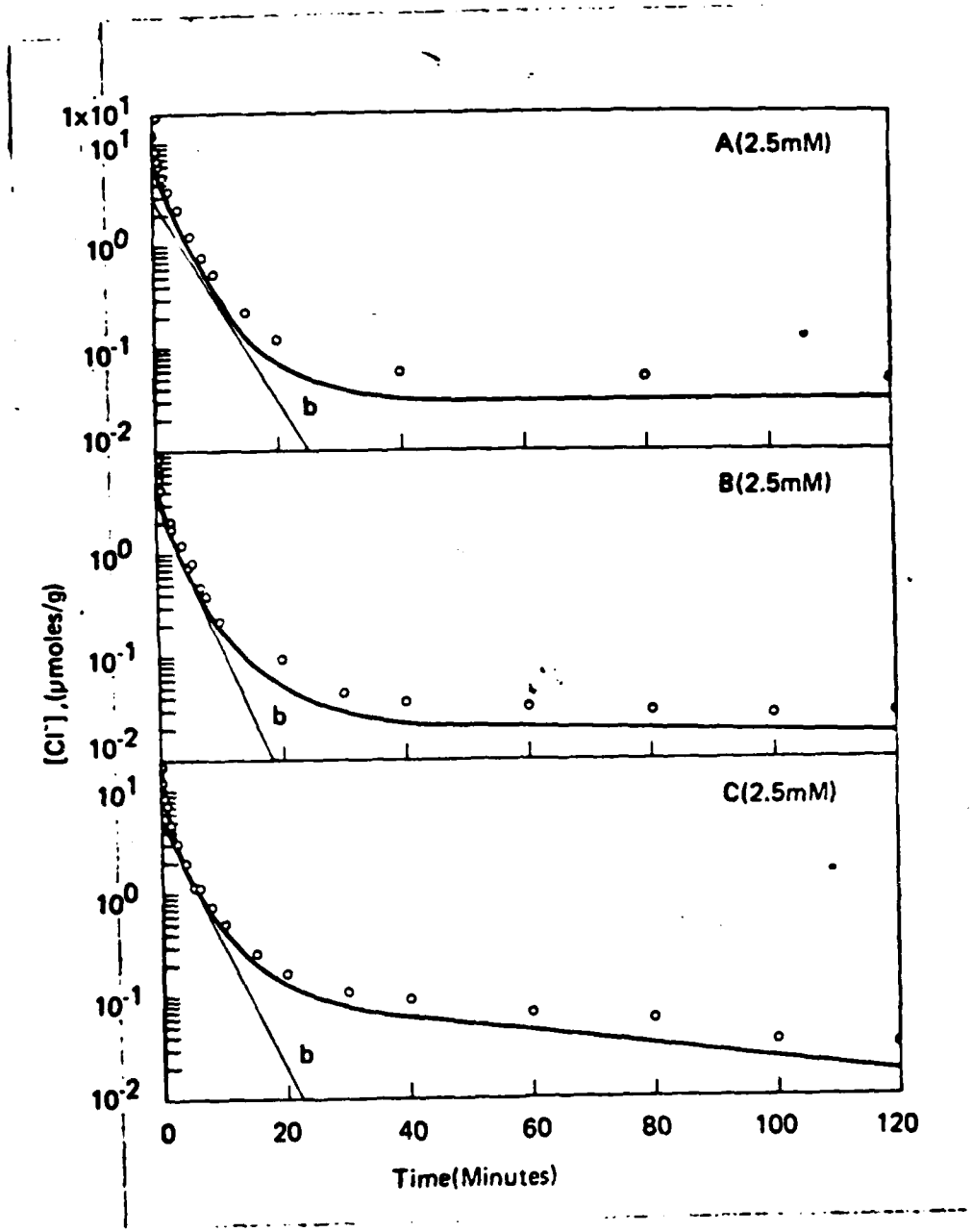

A(2.5mM)
B(2.5mM)
C(2.5mM)
b
[Cl⁻], (μmoles/g)
1x10¹
10¹
10⁰
10⁻¹
10⁻²
0
20
40
60
80
100
120
Time(Minutes)

FIGURE 3

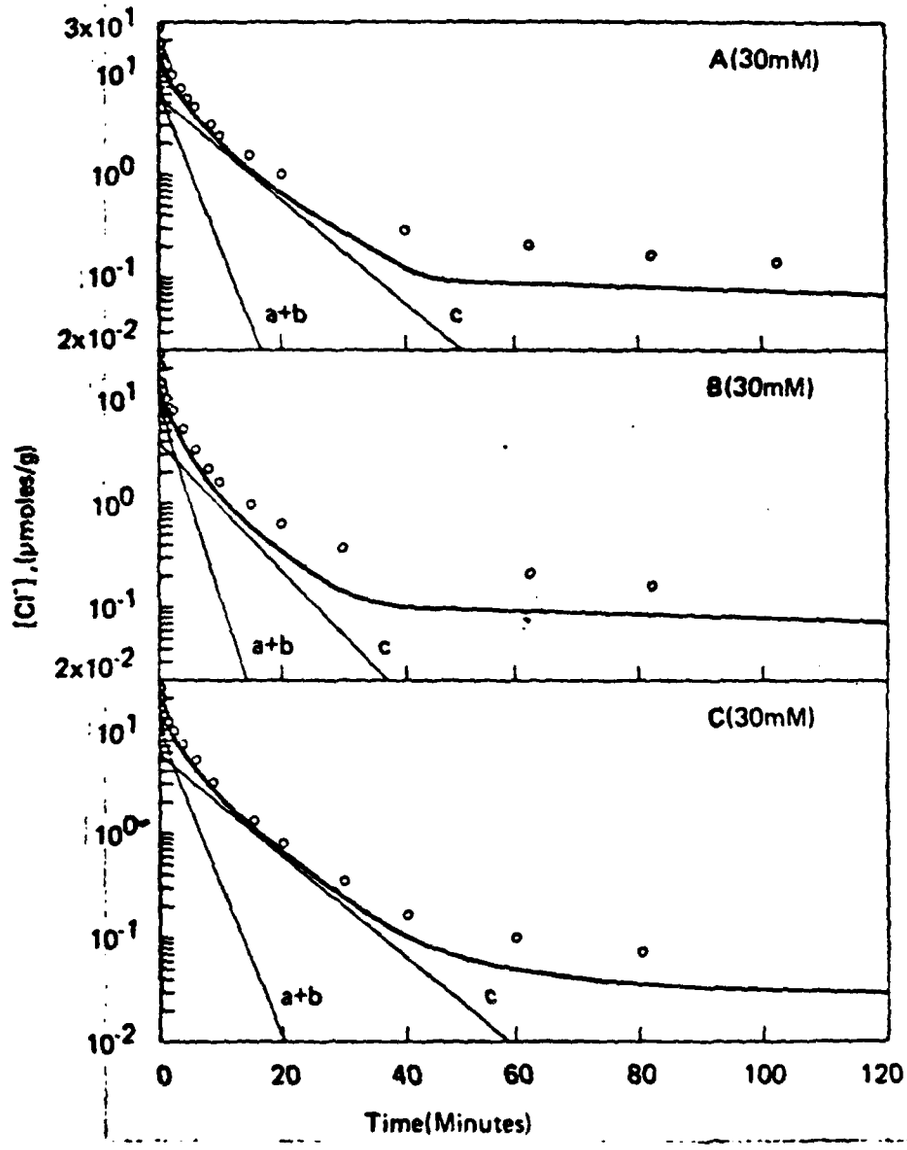

A(30mM)
B(30mM)
C(30mM)
a+b
c
[Cl⁻], (μmoles/g)
Time(Minutes)
3x10¹
10¹
10⁰
10⁻¹
2x10⁻²
10⁻²
0
20
40
60
80
100
120

FIGURE 4

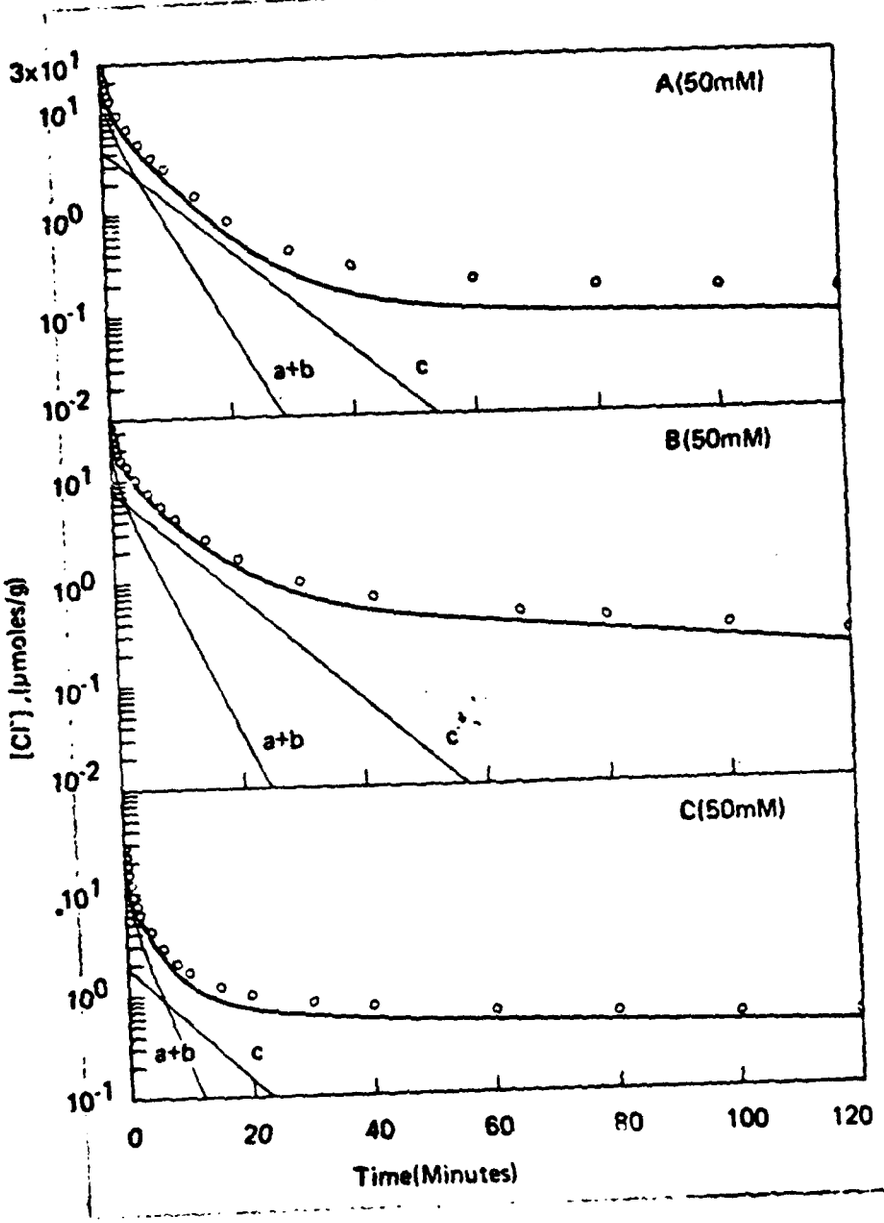

A(50mM)
B(50mM)
C(50mM)
a+b
c
[Cl⁻], μmoles/g
3x10¹
10¹
10⁰
10⁻¹
10⁻²
0
20
40
60
80
100
120
Time(Minutes)

FIGURE 5

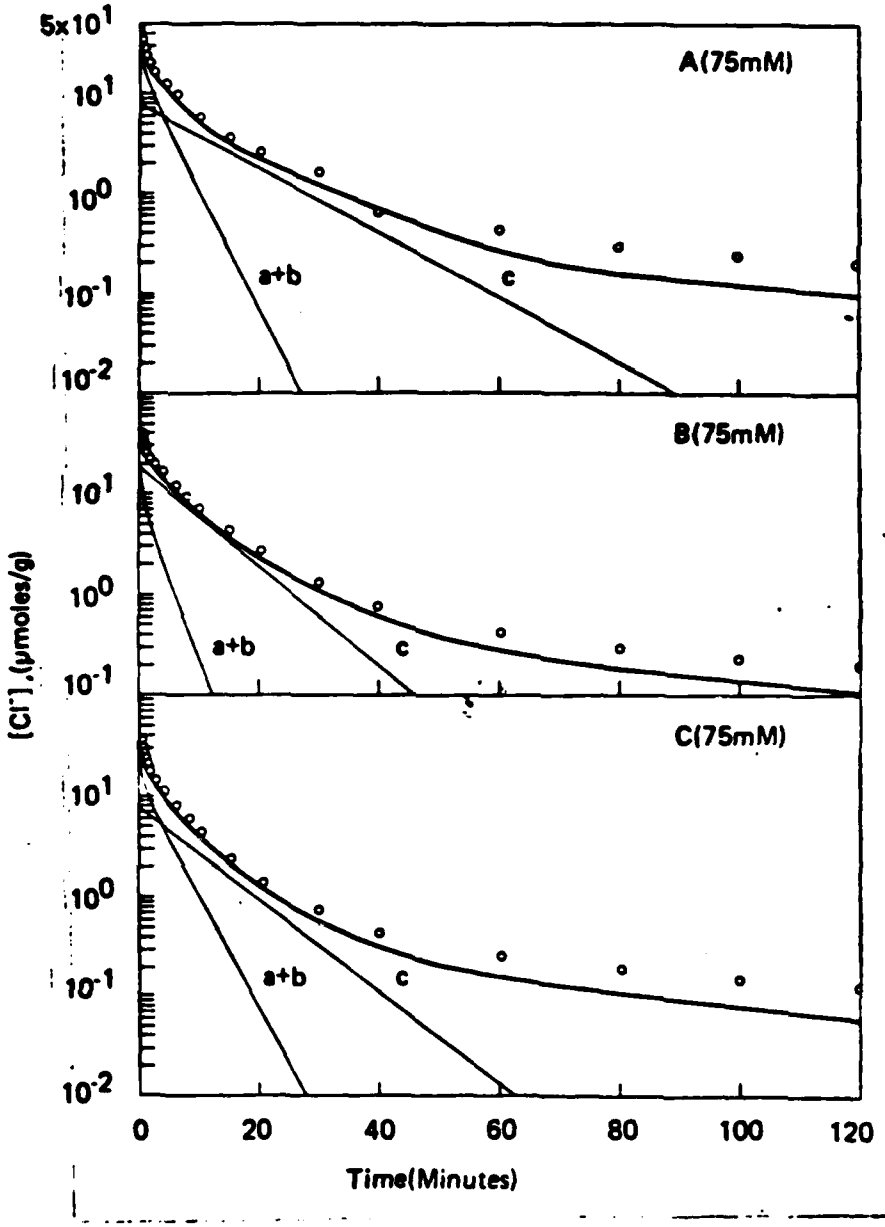

A(75mM)
B(75mM)
C(75mM)
a+b
c
[Cl⁻], (μmoles/g)
5x10¹
10¹
10⁰
10⁻¹
10⁻²
0
20
40
60
80
100
120
Time(Minutes)

FIGURE 6

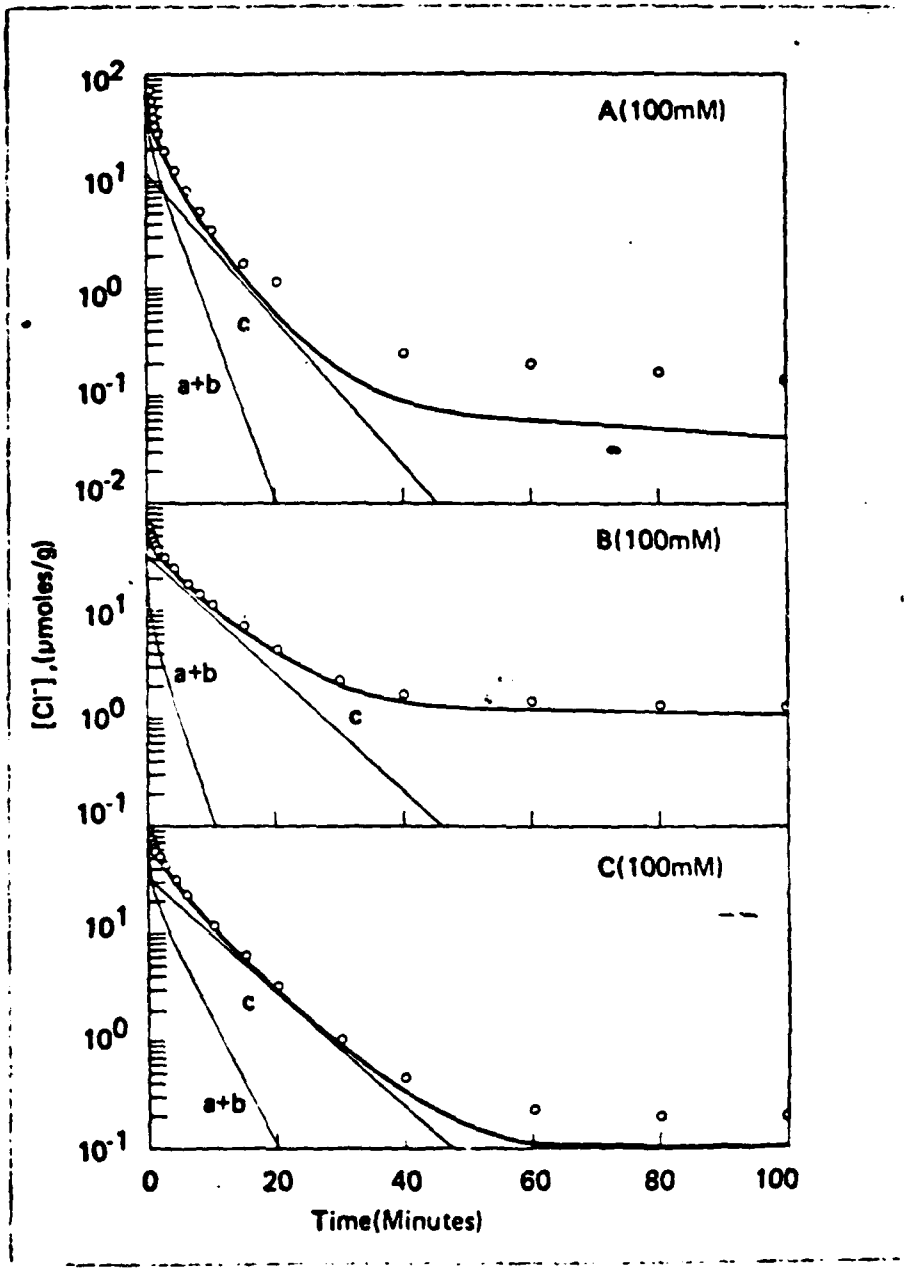

A(100mM)
B(100mM)
C(100mM)
a+b
c
[Cl⁻], (μmoles/g)
Time(Minutes)
10²
10¹
10⁰
10⁻¹
10⁻²
0
20
40
60
80
100

FIGURE 7

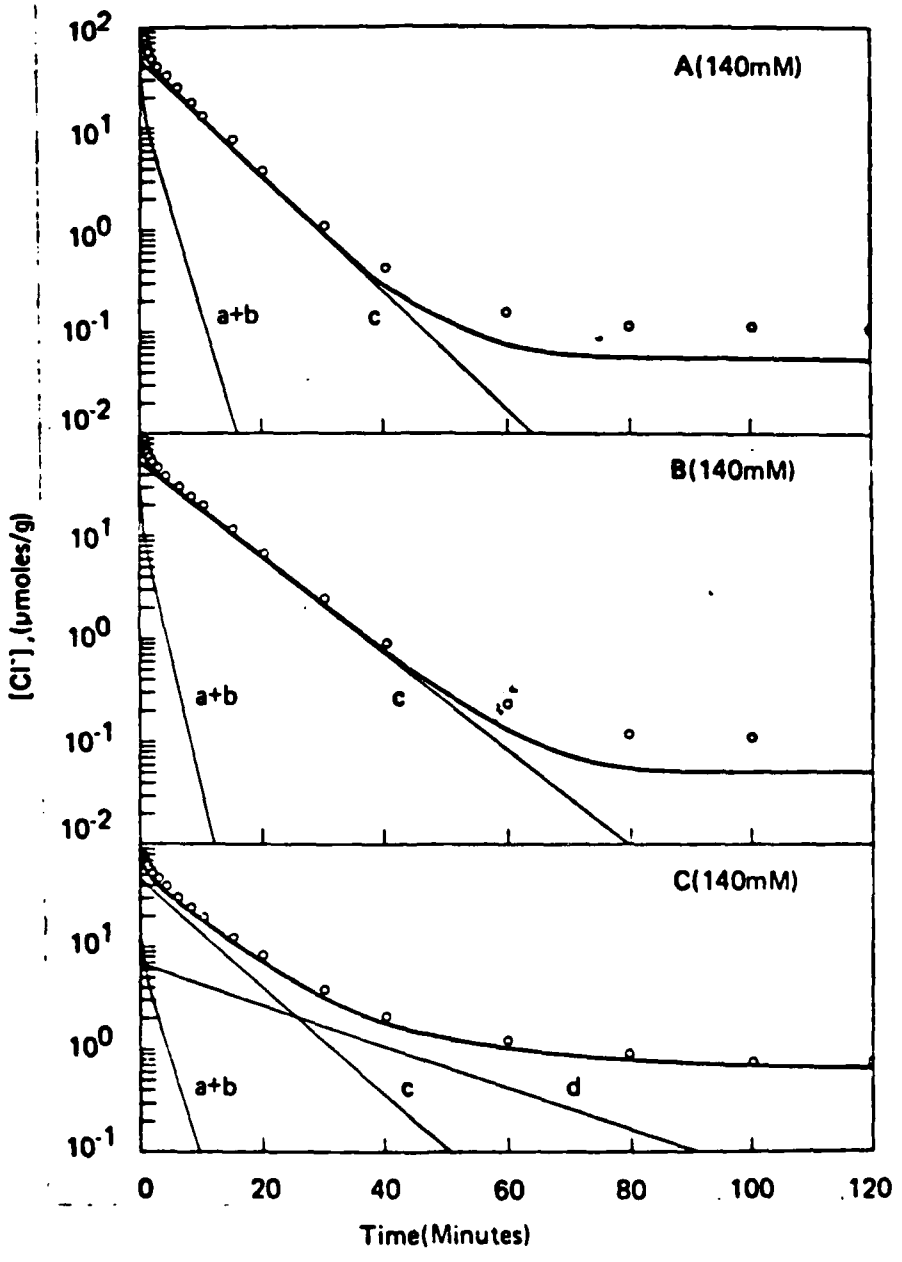

A(140mM)
B(140mM)
C(140mM)
a+b
c
d
[Cl⁻], (μmoles/g)
Time(Minutes)
0
20
40
60
80
100
120

FIGURE 8

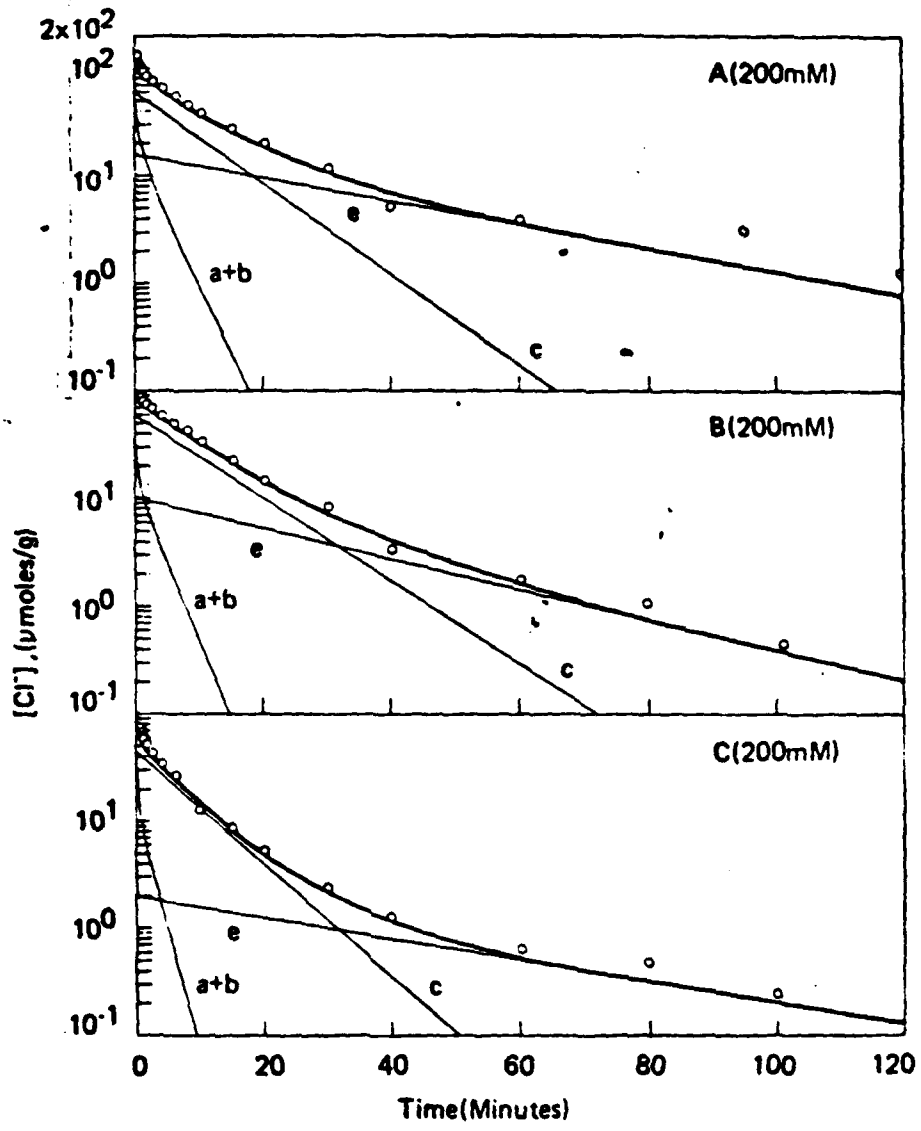

A(200mM)
B(200mM)
C(200mM)
a+b
c
e
[Cl⁻], (μmoles/g)
Time(Minutes)
2x10²
10²
10¹
10⁰
10⁻¹
0
20
40
60
80
100
120

FIGURE 9

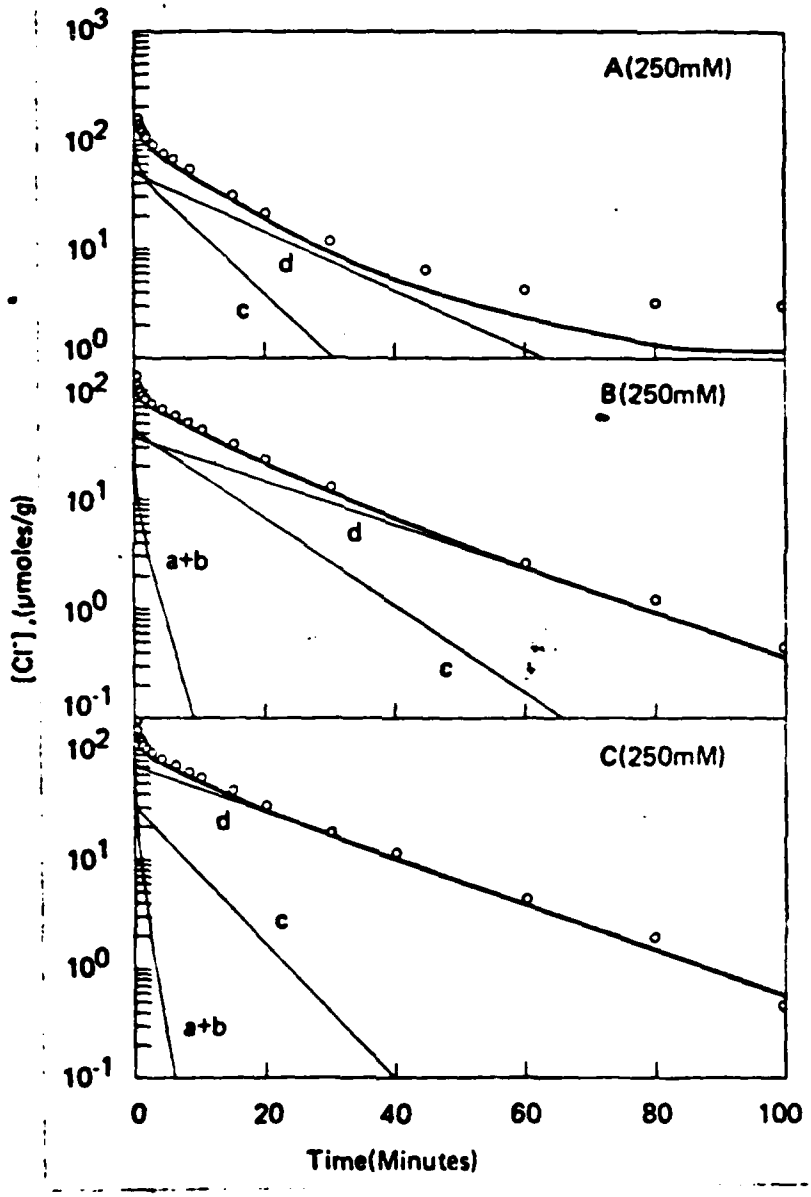

A(250mM)
B(250mM)
C(250mM)
d
c
a+b
10^3
10^2
10^1
10^0
10^{-1}
[Cl⁻], (μmoles/g)
0
20
40
60
80
100
Time(Minutes)

FIGURE 10

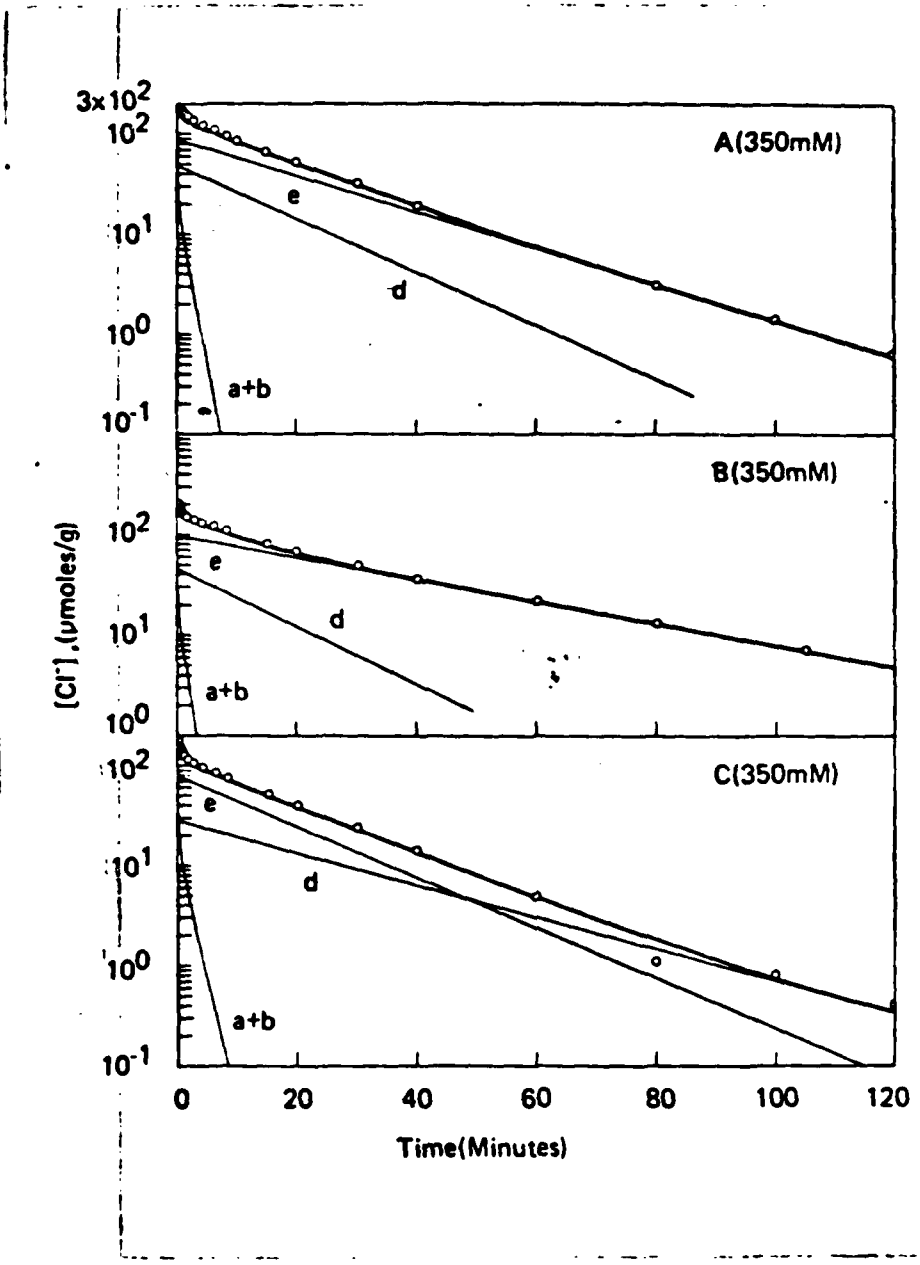

A(350mM)
B(350mM)
C(350mM)
e
d
a+b
[Cl⁻], (μmoles/g)
Time(Minutes)
0
20
40
60
80
100
120

FIGURE 11

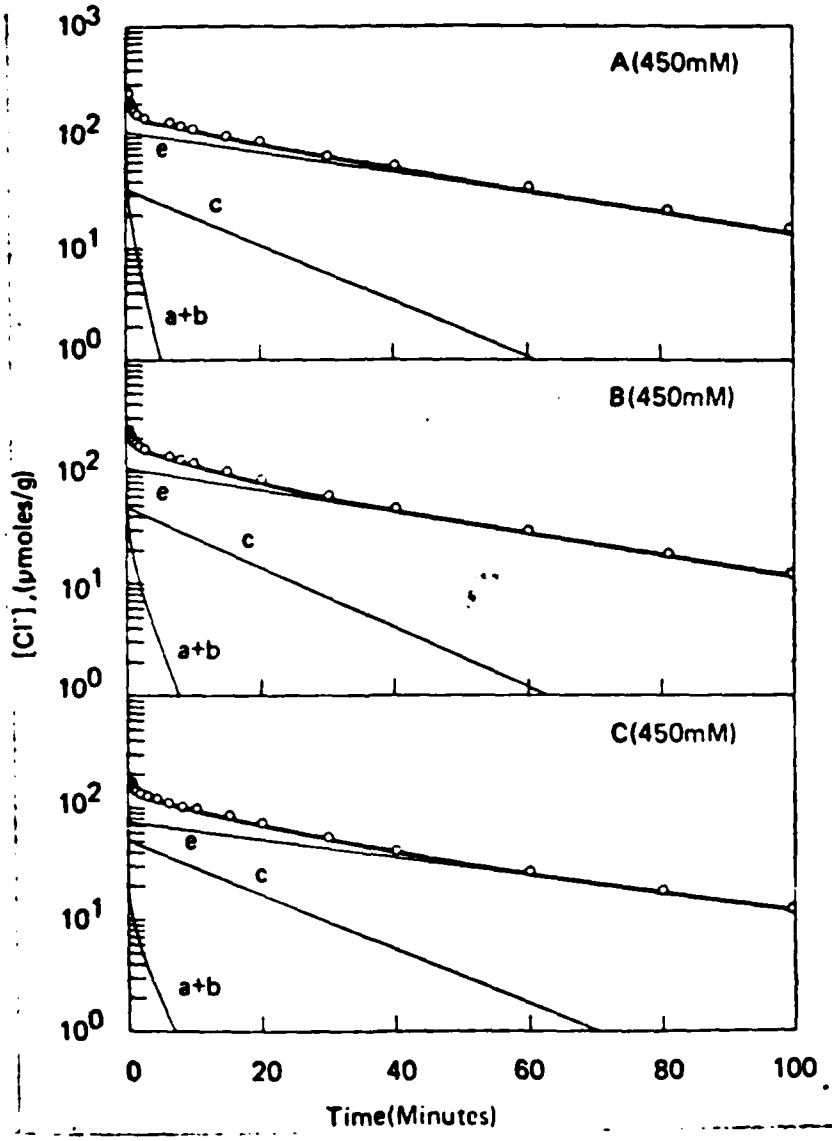

A(450mM)
B(450mM)
C(450mM)
e
c
a+b
10^3
10^2
10^1
10^0
[Cl⁻], (μmoles/g)
0
20
40
60
80
100
Time(Minutes)

FIGURE 12

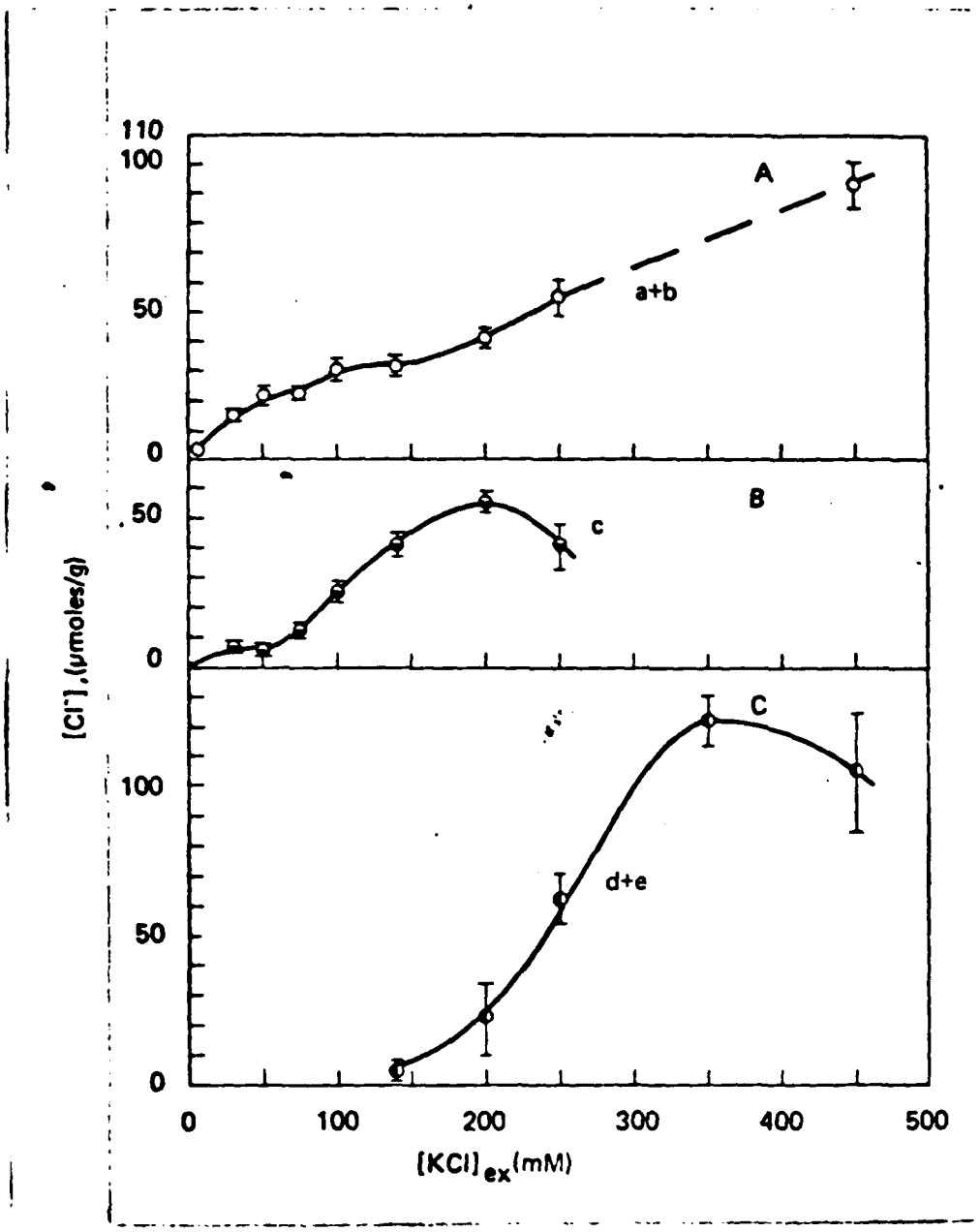

110
100
50
0
A
a+b
50
0
B
c
[Cl⁻], (μmoles/g)
100
50
0
C
d+e
0
100
200
300
400
500
[KCl]ex (mM)

FIGURE 13

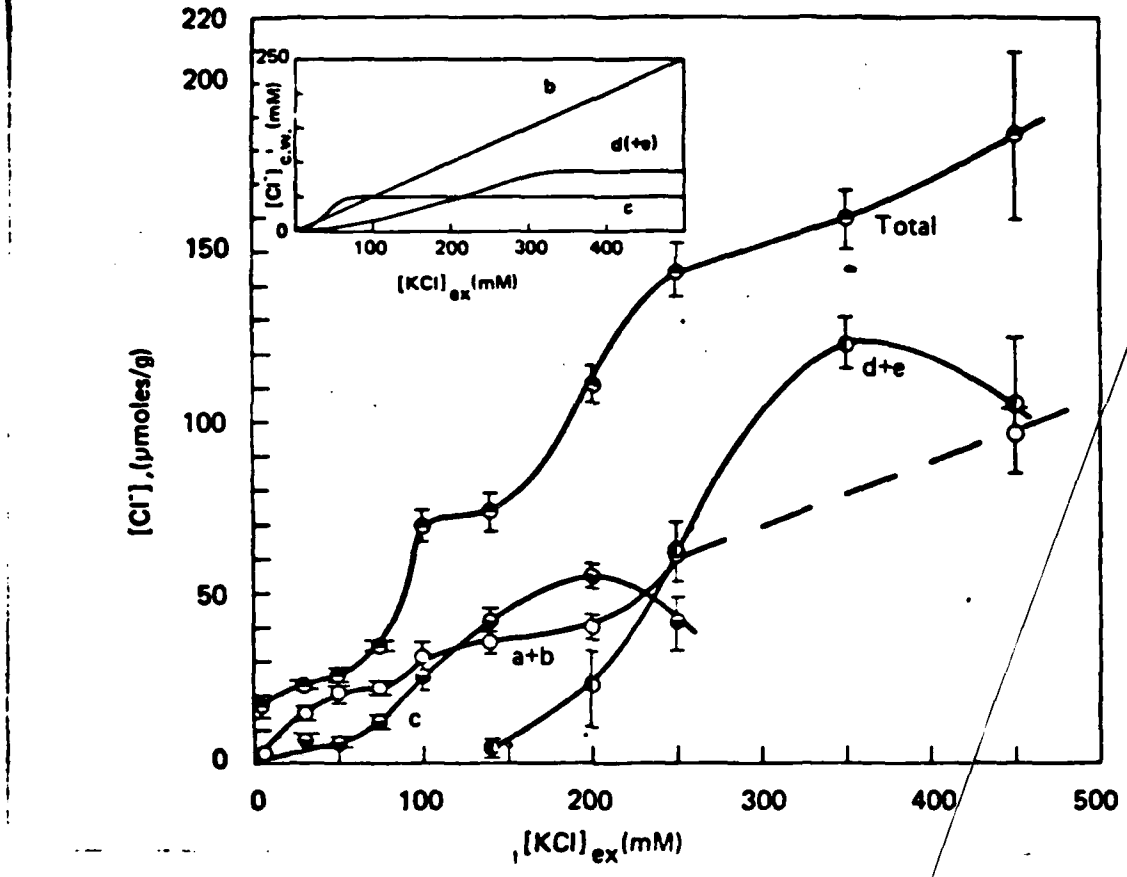

220
200
150
100
50
0
[Cl⁻], (μmoles/g)
0
100
200
300
400
500
[KCl]ex(mM)
Total
d+e
a+b
c
250
[Cl⁻]c.w. (mM)
b
d(+e)
c
100
200
300
400
[KCl]ex(mM)

FIGURE 14

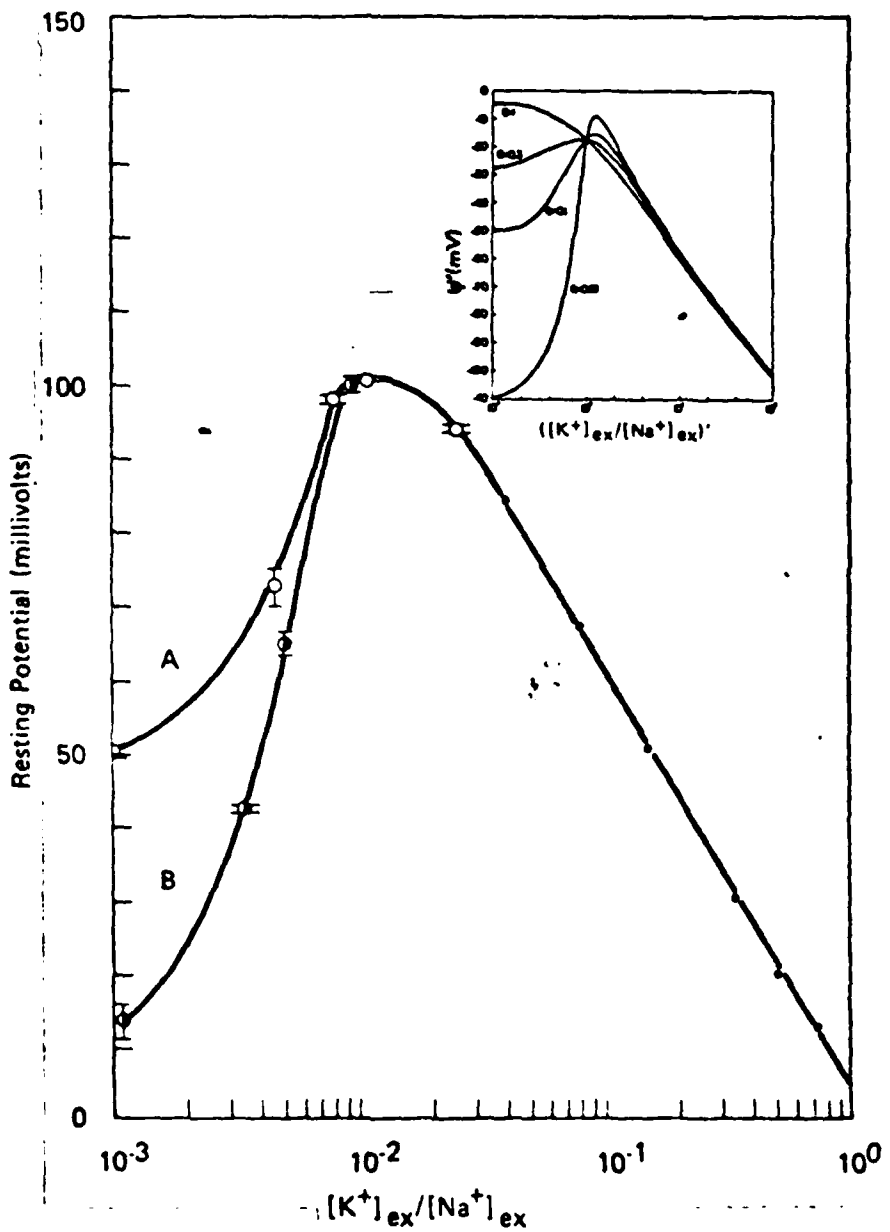

150
100
50
0
Resting Potential (millivolts)
A
B
10^{-3}
10^{-2}
10^{-1}
10^{0}
$[K^+]_{ex}/[Na^+]_{ex}$
ψ'(mV)
$([K^+]_{ex}/[Na^+]_{ex})'$

FIGURE 15